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The Role of Thymine-DNA Glycosylase In Transcriptional Regulation

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Abstract

Precise control over transcriptional regulation is required for normal cell function. Errors in transcriptional regulation underpin many diseases including cancer. Thymine DNA Glycosylase (TDG) is a base excision repair protein and a coregulator that has been implicated in a diverse set of fundamental biological processes including embryonic development, nuclear receptor signaling and Wnt signaling. Importantly, TDG has been shown to play an important role in transcriptional regulation in a wide variety of systems. Details surrounding the mechanism through which TDG acts remain unclear. In this thesis I explore the role of TDG in Estrogen Receptor (ER)-dependent signaling and in cellular senescence.

To characterize the role of TDG in ERα mediated signaling I first mapped β-Estradiol (E2)-dependent DNA binding of TDG in the MCF7 breast cancer cell line using ChIP-Seq. Using bioinformatics in conjunction with more traditional biochemistry techniques I established that a significant component of TDG binding occurs at enhancers, where it was able to mediate the production of enhancer RNA (eRNA) and 3-dimensional reorganization of transcriptional units. Knockdown of TDG disrupts E2-mediated upregulation of ER-targets and inhibits growth. Remarkably, in addition to behavior mimicking that of an oncogene, I find that TDG knockdown and depletion result in a much more aggressive phenotype, revealing its role as a potential potent tumor suppressor.

To explore the role of TDG in cellular senescence I induced senescence in IMR90 human fibroblasts using hydrogen peroxide (H_2O_2) and monitored markers of senescence,

including proliferation and β-galactosidase staining. I found that while senescence was readily inducible in this cell line using H_2O_2, knockdown of TDG was able to significantly impede the process. Using ChIP, I found that TDG was recruited to a CpG island overlapping the CDKN2A promoter, a tumor suppressor important for senescence. Further studies including ChIP, bisulfite sequencing and conventional assays revealed that TDG is required for H_2O_2-mediated transcription of CDKN2A in a CBP-dependent and active-demethylation independent manner.

Collectively, these studies extend the role of TDG in transcriptional regulation, implicating it as a mediator of cellular senescence and as a mediator of eRNA transcription and 3-dimensional re-organization in hormone signaling.

Keywords

Thymine DNA Glycosylase, Estrogen signaling, Senescence, Cancer, Enhancers, eRNA.

Co-Authorship Statement

All chapters were written by Bart Kolendowski and edited by Dr. Joe Torchia.

Chapter 2: Experiments were conceived by Bart Kolendowski and Dr. Joe Torchia. All bioinformatic analysis, E2 treatments, siRNA mediated knockdowns, growth curves, 3C, mRNA and eRNA qPCR were performed by Bart Kolendowski. Original E2 ChIP in MCF7 cells was performed by Gobi Thillainadesan and Majdina Isovic. CRISPR-MCF7 KO cell line was generated by Haider Hassan and Majdina Isovic. Haider Hassan performed validations and assisted with MAB-Seq and bisulfite sequencing. Milica Krstic performed the invasion, migration and cell-to-cell adhesion assays. Dr. Ann F. Chambers and Dr. Alan B. Tuck assisted with experimental design and were involved with editing and finalizing the manuscript. A version of chapter 2 has been published (Kolendowski et al., 2018).

Chapter 3: Experiments were conceived by Bart Kolendowski and Dr. Joe Torchia. All H_2O_2, growth curves, Western blot analysis, siRNA treatments, bisulfite sequencing, β-galactosidase staining, and downstream bioinformatic analysis were performed by Bart Kolendowski. Haider Hassan assisted with TDG and CBP ChIP-qPCR. Majdina Isovic set up the timed matings, isolated mouse embryos and assisted in double blind study.

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List of Abbreviations

3C	Chromosome conformation capture
4-OH-TAM	4-Hydroxytamoxifen
5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
AID	Activation-induced deaminase
AID	Activation induced cytidine deaminase
AP	Apurinic site
APE1	apurinic/apyrimidinic (AP) endonuclease 1
APOBEC	Apolipoprotein B mRNA editing catalytic polypeptide-like
APOBEC 1-4	Apolipoprotein B mRNA editing enzymes
BER	base excision repair pathway
CBP	CREB-binding protein
ChIA-PET	Chromatin Interaction Analysis by Paired-End Tag sequencing

CpG	Cytosine-Guanine dinucleotide
CRC	colorectal cancer
DBD	DNA-binding domain
DNMT	DNA methyltransferases
E2	β-estradiol
ER	estrogen receptor
eRNA	enhancer RNAs
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FBS	Fetal Bovine Serum
FGF1	Fibroblast Growth Factor 1
Flox	Flanking/flanked by LoxP sites
GADD45	Growth arrest and DNA damage 45
GO	Gene Ontology
GRO-Seq	Global Run-On Sequencing
H_2O_2	Hydrogen Peroxide

MAB-Seq	Methylase-assisted bisulfite sequencing
mESC	mouse Embryonic Stem Cell
MUG	Mismatch Uracil Glycosylase
NR	Nuclear Receptors
PBS	Phosphate Buffered Saline
PIC	pre-initiation complex
qPCR	Realtime PCR
SASP	Senescence Associated Secretory Phenotype
SRC	steroid receptor coactivators
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCGA	The cancer genome atlas
TDG	Thymine DNA glycosylase
TET	Ten Eleven Translocation
UDG	Monofunctional Uracil-DNA Glycosylase
WNT	Wingless-type MMTV integration site

Chapter 1. Introduction

1.1 Overview

Multicellular organisms are composed of a variety of cell types that perform specialized functions critical to an organism's survival. Diverse cell types within the same organism often contain the same set of DNA yet can exhibit very different morphologies and functions. This discrepancy is resolved by the observation that a cell's phenotype is controlled not by the set of genes it possesses, but rather by which genes are expressed: transcribed into RNA and ultimately translated into protein. As such, cells of the same "type" often share expression profiles that closely resemble one another but differ from cells of a different type. Changes to a normal cell's expression profile can result in changes to its established function and morphology. When this occurs more generally, an organism's fitness can become impacted. For example, during embryonic development, where cells progress from undifferentiated stem cells to differentiated cells with specific functions, control of transcription is particularly critical and disruption to the transcriptional program can result in developmental errors that can be lethal (Cortázar et al., 2011; Cortellino et al., 2011; Li et al., 1992). Loss of transcriptional regulation and subsequent disruption of expression patterns is also observed to underlie numerous pathologies. A consistent feature found in many cancers is that the transcription of tumor suppressors and oncogenes are often dysregulated (Bihl et al., 2012; Frietze et al., 2014; Zheng and Blobel, 2010). Therapeutically, targeting defects in expression in order to re-establish 'normal' expression patterns has been used successfully to treat some diseases: in the case of cancer these act primarily by silencing oncogenes or re-establishing expression of some tumor suppressors (Morris and Chan, 2015). Building a deeper

understanding of transcriptional regulation may provide insight into pathologies with an underlying dysregulation of transcription.

An important component of transcriptional regulation are the coregulators which can further be subdivided into “coactivators” and “corepressors”. Coregulators are proteins that modulate transcription of DNA by controlling the transcriptional machinery’s accessibility to the DNA. This is achieved either directly, by altering the chromatin structure through covalent modification to the DNA and/or associated histones, or indirectly, acting as scaffolds which then recruit other proteins that can then modify the chromatin. Thymine DNA Glycosylase (TDG) is a coregulator that has been implicated in a diverse set of fundamental biological processes including embryonic development, nuclear receptor signaling and Wnt signaling. While TDG has been shown to regulate transcription in a wide variety of systems, details surrounding its mechanism of action remain unresolved. In this thesis I seek to extend our knowledge of transcriptional regulation by exploring the role of TDG in different contexts. Specifically, I investigate TDG’s role in estrogen receptor dependent signaling and in cellular senescence.

1.2 Eukaryotic Transcriptional Regulation

The establishment and maintenance of correct expression profiles is required by all biological process from development to fully differentiated tissues. Deviations from normal expression patterns are often observed in cells during disease development and can be directly responsible for the pathological changes to a cell’s phenotype. Transcription of DNA to its corresponding RNA and finally translation to the protein product is a highly

regulated process with multiple checkpoints along the way. Because it requires a considerable investment of energy and resources, regulation during the initial stages is much more efficient as it prevents the cell from unnecessarily wasting resources and energy. One of the earliest points of regulation occurs during the transcription of DNA to RNA. Control at the transcriptional level can be broadly categorized into 2 distinct, but intimately related mechanisms: Transcription factor dynamics at regulator regions as well as epigenetic control over accessibility to these regions.

1.2.1 Transcriptional Regulatory Regions

Transcription occurs through 3 distinct stages:

(1) Initiation - the localization and assembly of critical transcriptional machinery, RNA Pol II and its auxiliary factors to the targeted gene's regulatory regions.

(2) Elongation - The process whereby RNA Pol II transverses the DNA template and assembles the RNA molecule.

(3) Termination – Dissociation of RNA Pol II from DNA template.

The initiation stage of transcription is a particularly important point of regulation as the cell has not yet invested energy or resources at this point. During this stage, the transcriptional machinery is typically localized to the gene's regulatory regions, which contain elements critical for its transcription. Transcriptional initiation requires the formation of a "pre-initiation complex" (PIC) that consists of, at a minimum, RNA Pol II (the enzyme responsible for synthesizing the RNA molecule from DNA template) and its

6 general auxiliary factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) (Luse, 2013). The promoter, a region of DNA adjacent to the gene and often encompassing the transcriptional start site, contains regions that facilitate the formation of this complex. The promoter often contains a B recognition element (BRE), a downstream promoter element (DPE) and the "initiator element" or a TATA box. Generally, the presence of the TATA box and initiator element is mutually exclusive: promoters tend to have one, but not both, of these elements. During transcriptional initiation, these highly conserved elements are recognized and bound by auxiliary factors (either TFIID or TFIIB) which then results in further sequential binding of the additional auxiliary factors and RNA Pol II itself, forming the PIC. While the PIC is sufficient to produce minimal levels of transcription, in order to achieve the levels that often observed *in vivo*, an additional class of molecules called 'transcription factors' must be recruited.

1.2.2 Transcription Factors

Transcription factors are proteins that regulate transcription at the point of transcriptional initiation, by facilitating or inhibiting loading of the PIC (Petrykowska et al., 2008). Conservative estimates put the number of transcription factors at approximately 1,300 (approximately 6% of the protein coding genes in the human genome) (Vaquerizas et al., 2009). Transcription factors can be broadly classified into two mechanistic categories: (1) General transcription factors which include RNA Polymerase II and its auxiliary factors that form the pre-initiation complex and which are ubiquitously expressed in tissues and (2) Sequence specific transcription factors which recognize and bind to specific sequences of DNA and which are frequently expressed in a tissue-specific fashion.

Structurally, specific transcription factors are often composed of multiple domains that each serve distinct functions:

(1) DNA binding domain - a structural motif often composed of alpha helices, beta sheets, and/or disordered regions that recognizes and binds specific DNA sequences termed 'response elements' or 'transcription factor binding motifs'. A DNA binding motif can take many forms, for example the most common motifs include the helix-loop-helix domain that is characterized by a simple structure of two α-helices linked by a loop, the "zinc finger" domain, whose activity and structure requires the presence of one or more zinc ions, as well as structurally more complex domains such as the "winged-helix transcription factors" which are composed of four helices and a two-strand beta-sheet (Yusuf et al., 2012).

(2) Signal sensing domain – This domain allows transcription factors to respond to the presence of endogenous or exogenous molecules by altering their activity and/or localization. A well-studied example of this is the Estrogen Receptor α (ER), a nuclear hormone receptor that underlies sexual and reproductive development in females. ERα is found mostly in the cytoplasm under normal conditions (Putnik et al., 2012). Upon binding β-Estradiol (E2), an ERα agonist, ERα molecules undergoes a conformational change, dimerizing and translocating into the nucleus where they bind to "Estrogen Response Elements", a specific DNA sequence recognized by ERα and found in the regulatory region of ER-target genes. Recruitment of additional factors follows ERα binding, resulting in transcriptional upregulation of the ERα target genes (Carroll et al., 2006; Hah et al., 2013; Welboren et al., 2009).

(3) Transactivation domain – In order to significantly impact transcription levels of a target gene, the recruitment of an additional class of proteins called “coregulators” is often required. The transactivation domain is a series of amino acids that function as a scaffold capable of recruiting certain coregulators. Both transcription factors and coregulators can have multiple interaction domains that allow for each to interact with multiple partners. For example, p53 contains two transactivation domains that are a part of the “Nine Amino Acid Transactivation Domain” a family of transactivation domains common to many eukaryotic transcription factors. This transactivation domain interacts with specific protein binding domains including the “TAZ1” and “KIX” domains which are both found on coregulators such as, CBP/p300. The interaction between these domains and those of p53 is required for CBP/p300 recruitment and activity at sites of p53 binding (Kasper et al., 2011; Lee et al., 2010).

Transcription factors can be regulated through transcriptional silencing and through post-translational modifications that can either enable or prevent them from binding to their respective response elements. This is important, as the transcription levels of a specific gene are often dictated not by a single transcription factor, but rather by the collective effects of the transcription factors bound to its regulatory region. Certain transcription factors are only present in particular cell types, allowing those cells to respond to a particular stimulus in a manner entirely distinct from cell types that expresses a different set of transcription factors. This “combinatorial system” of transcriptional regulation allows an organism to respond to a vast and diverse set of exogenous and endogenous cues, while limiting the required genome size and the amount of transcription factors needed (Reményi et al., 2004; Vaquerizas et al., 2009).

1.2.3 Coregulator-mediated regulation of chromatin

In eukaryotic cells, DNA is stored in a complex with protein and RNA called ‘chromatin’ (Figure 1-1). The fundamental subunit of chromatin is the nucleosome consisting of approximately 147 base pairs (bp) of DNA wrapped around an octamer of positively charged histone proteins containing two of each of H3, H4, H2A and H2B (Eickbush and Moudrianakis, 1978). Nucleosomes are separated by short stretches of ‘linker’ DNA approximately 80 bp in length. Chromatin at this level of organization is referred to as ‘euchromatin’ and when viewed under an electron microscope resemble ‘beads on a string’ (Figure 1-2) (Cann and Dellaire, 2011). The next level of organization involves “linker” histone H1, a non-core histone protein that binds outside of the core nucleosome at the location where the DNA enters/exits the nucleosome and that also interacts with the region of linker DNA that connect histones. Histone H1 interactions stabilize the DNA into a further compacted, “30nm fiber” (debate however still exists as to the specific structure this fiber takes) (Cann and Dellaire, 2011). Chromatin at this level of organization is often referred to as heterochromatin. Chromatin generally exists in a heterogenous state composed of both euchromatin and heterochromatin, however during specific stages of the cell cycle (i.e. mitosis/meiosis), chromatin is further compacted into chromosomes in preparation for cellular division.

Figure 1-1. Progressive levels of chromatin compaction.

Incorporation of linker histone H1 generally marks the transition from a euchromatin (favorable to transcription) to heterochromatin (transcriptionally repressed state).

(With modification from source:

https://commons.wikimedia.org/wiki/File:Chromatin_Structures.png)

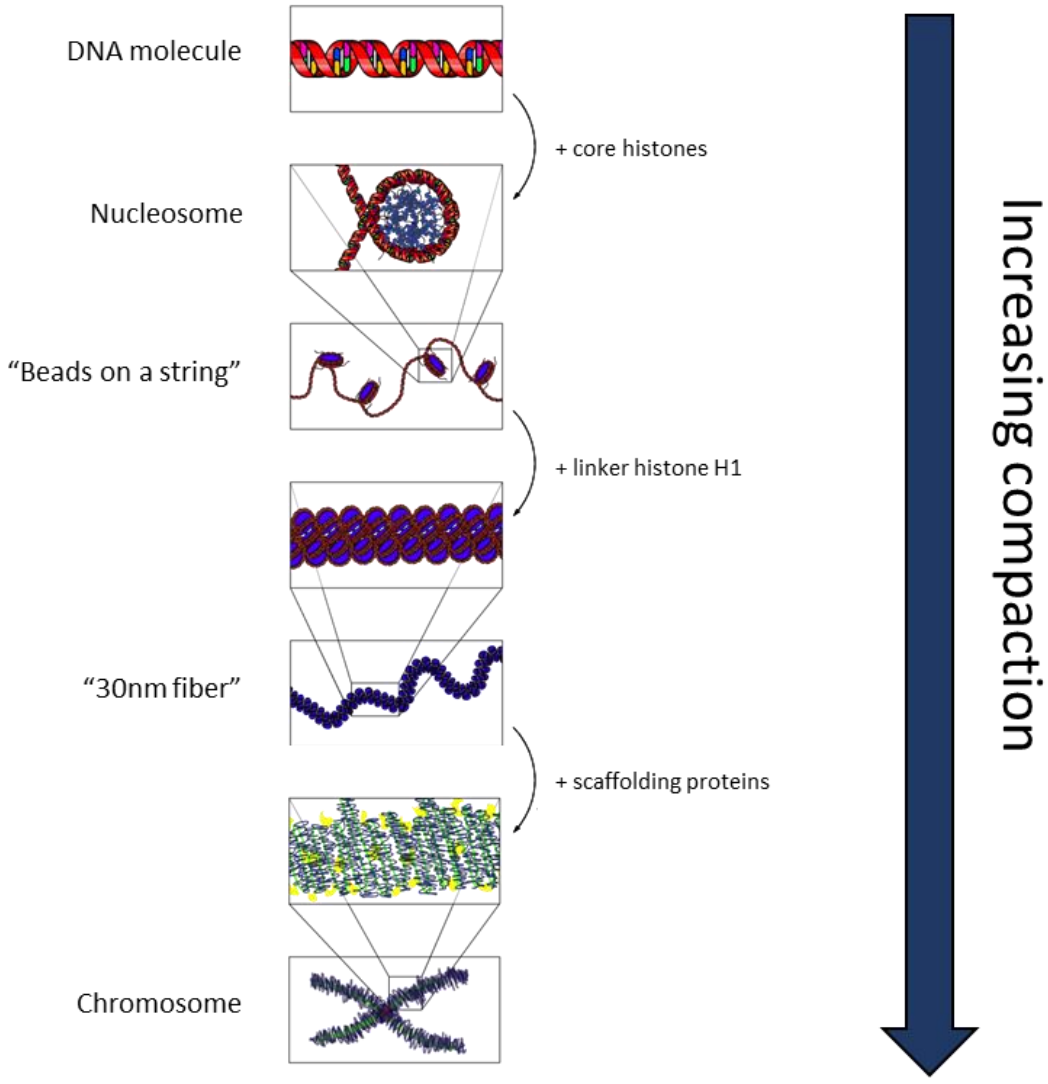
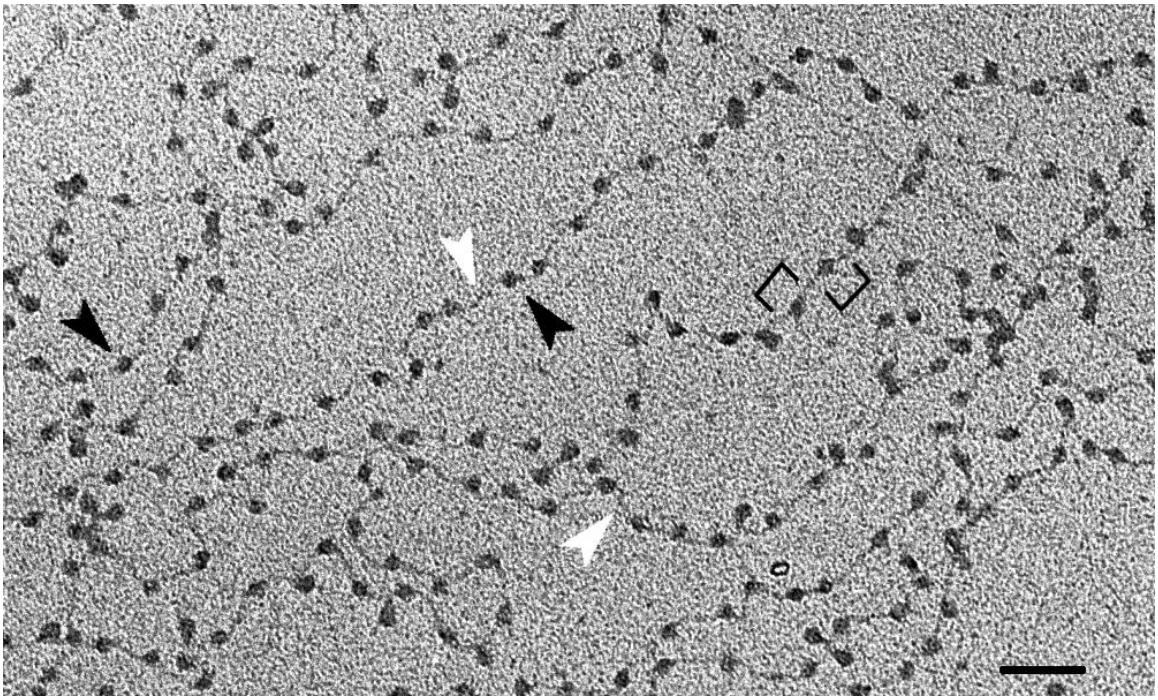

DNA molecule
+ core histones
Nucleosome
"Beads on a string"
+ linker histone H1
"30nm fiber"
+ scaffolding proteins
Chromosome
Increasing compaction

Figure 1-2. Electron micrograph of '11nm chromatin fiber'.

Also referred to as "beads on a string" the chromatin in this state is composed of nucleosome units (black brackets), that can be further resolved to nucleosomes (black arrowhead) and DNA linker region (white arrowhead). (Original image by Chris Woodcock, doi:10.7295/W9CIL709)



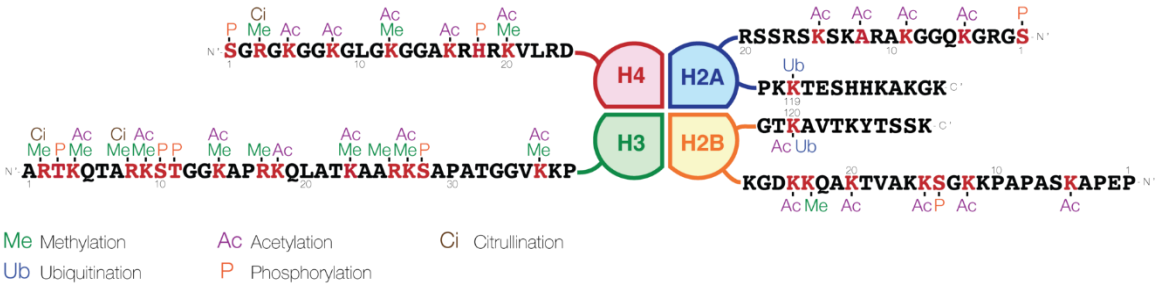
Chromatin structure is inherently linked to transcriptional activity: the 'relaxed' organization of euchromatin readily allows the transcriptional machinery to gain access to DNA and is therefore often found in regions that are transcriptionally active. Heterochromatin, which is a highly compacted structure, masks the underlying DNA from the transcriptional machinery, inhibiting access to general and most sequence specific transcription factors, and is therefore often found associated with transcriptionally silent areas of the genome. An exception to this is a class of transcription factors called 'pioneer factors' which possess a certain structure that allows them to bind heterochromatinized DNA. Once bound to DNA, transcription factors often recruit another class of proteins, called 'coregulators', that dynamically alter the chromatin landscape, either making it more or less permissible to transcription. Therefore, whether a transcription factor has a positive or negative effect on transcriptional activity is often dictated by the coregulators it recruits.

Coregulators can alter the structure of chromatin primarily through two methods: (1) The removal of nucleosomes through an ATP-dependent process, and/or (2) post-translational modifications of histones. Post-translational modifications often occur in the form of covalent addition of an acetyl, methyl or phosphoryl group to specific residues on unorganized regions of the histone that protrude out from the nucleosome referred to as histone 'tails' (Figure 1-3) (Eickbush and Moudrianakis, 1978). The addition or removal of these modifications can alter the affinity between the DNA and histones which leads to either a relaxing or tightening of the chromatin structure that, in turn, impacts transcription levels. In the case of histone acetylation for example, addition of acetyl groups to specific lysine residues on the histone tails neutralizes the positive charge on the histones disrupting their affinity for negatively charged DNA.

Figure 1-3. Histone tail modifications.

Schematic highlighting potential histone tail modifications that have been observed. Generally, only a subset of these will be present on any one nucleosome. At any one particular site acetylation and methylation can be found to be mutually exclusive, targeting the same residue.

(Source: Wikimedia Commons. Author: Mariuswalter; Based on work from Rodriguez-Paredes and Esteller, Nature, 2011)


H4
H2A
H3
H2B
Me Methylation
Ac Acetylation
Ci Citrullination
Ub Ubiquitination
P Phosphorylation

This weakened affinity promotes an ‘open’ chromatin state, encouraging transcriptional machinery access. Coregulators that catalyze this reaction, called histone acetyltransferases, are therefore often referred to more specifically as “coactivators” as their presence strongly corresponds to increased transcriptional activity. In contrast, coregulators that contain histone deacetylase activity, remove acetyl groups from histones, and therefore exert the opposite effect: they reestablish the positive charge on histones which increases the affinity for DNA promoting a more organized chromatin structure that is less accessible to the transcriptional machinery. Coregulators with histone deacetylase activity are therefore often referred to as “corepressors” and are often found at transcriptionally silenced genomic regions.

The relationship between histone methylation and transcription is more complex because methylation of specific residues can correspond to either transcriptional silencing or activation depending on the location and the abundance of methyl marks that are deposited. For example, trimethylation of lysine 27 on Histone H3 (H3K27me3), which is catalyzed by EZH2, a component of the polycomb repressive complex 2, is found exclusively at transcriptionally silenced regions, while monomethylation of the same site (i.e. H3K27me1) is strongly associated with active transcription (Barski et al., 2007). Importantly, genomic regions contain numerous nucleosomes that are each composed of multiple histones, and each histone has multiple sites that can be modified. While certain histone modifications are strongly correlated with the expression or repression of any given region, it is the specific permutation of histone modifications (i.e. the “Histone code”) that best correlates with the coactivator composition at that region and its transcriptional status. In addition to altering the affinity between the DNA and histones, addition or removal of a

specific modification from a histone residue can also create binding sites for factors that can recognize a particular modification or, conversely, mask such a site – further complicating the effects that covalent modifications may have in any given context.

1.2.4 DNA Methylation and Active Demethylation

In addition to changes in the organization of chromatin, modifications to the DNA molecule, primarily in the form of covalent modifications to cytosine, can have profound effects on transcriptional regulation (Aran and Hellman, 2013). Cytosine can be covalently modified primarily in a CpG context. DNA methylation patterns are maintained by DNA methyltransferases (DNMTs) which recognize unmethylated/hemi-methylated DNA and catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to the C5 carbon of cytosine, creating 5-methylcytosine (5mC). Remarkably, while most CpG's in normal tissue are methylated, promoters often contain long stretches of CpGs (CpG islands) which are maintained in an un-methylated state (Feltus et al., 2003). Hyper-methylation of these promoters is strongly associated with transcriptional repression of the associated gene(s) (Baylin and Herman, 2000). Mechanistically, 5mC can repress transcription either directly by making regulatory sites inaccessible to transcription factor binding, or indirectly through the recruitment of proteins that contain methyl-binding domains which in turn, recruit additional proteins which have repressor activity.

Patterns of global methylation and promoter-specific hypomethylation are maintained in healthy tissue (Baylin and Herman, 2000). However, in most cancers DNA methylation is highly dysregulated consisting of global hypomethylation and promoter-specific

hypermethylation. Global hypomethylation has been proposed to have an oncogenic effect by activating epigenetically silenced transposable elements or endogenous viruses, as well as increased rates of chromosomal rearrangements (Chen et al., 1998b). Furthermore, many tumor-suppressor genes contain CpG islands in their promoters that are hypermethylated in cancer and are transcriptionally silenced. Importantly, DNA methylation inhibitors can reactivate epigenetically silenced tumor suppressors and have been used therapeutically (Licht, 2015). While the mechanism governing DNA methylation has been extensively researched and is well characterized, the mechanism(s) governing DNA demethylation have proven to be more elusive.

DNA demethylation can occur through both active and passive pathways. The passive pathway is achieved primarily through the loss of DNMT activity. In oocytes this is achieved primarily through the exclusion of DNMT1 from the nucleus, while in primordial germ cells this is achieved through the down-regulation of UHRF1, a protein that targets DNMT1 to hemi-methylated DNA (Messerschmidt et al., 2014; Ratnam et al., 2002). During proliferation, this inability to methylate DNA results in the gradual dilution of the methylation signal over successive rounds of DNA replication. On average, cells would theoretically retain 50% of the number of methylated CpG's that the previous generation had, meaning that after 9 cell divisions the retained percentage of methylation remaining would be approximately 0.2% of the first generation's methylation levels.

The first clues that an "active" demethylation process must exist came from the observation that paternal DNA is almost entirely (80-90%) methylated prior to zygote formation, yet it becomes entirely de-methylated shortly thereafter, prior to DNA replication (Messerschmidt et al., 2014). Subsequent work has found that active demethylation is

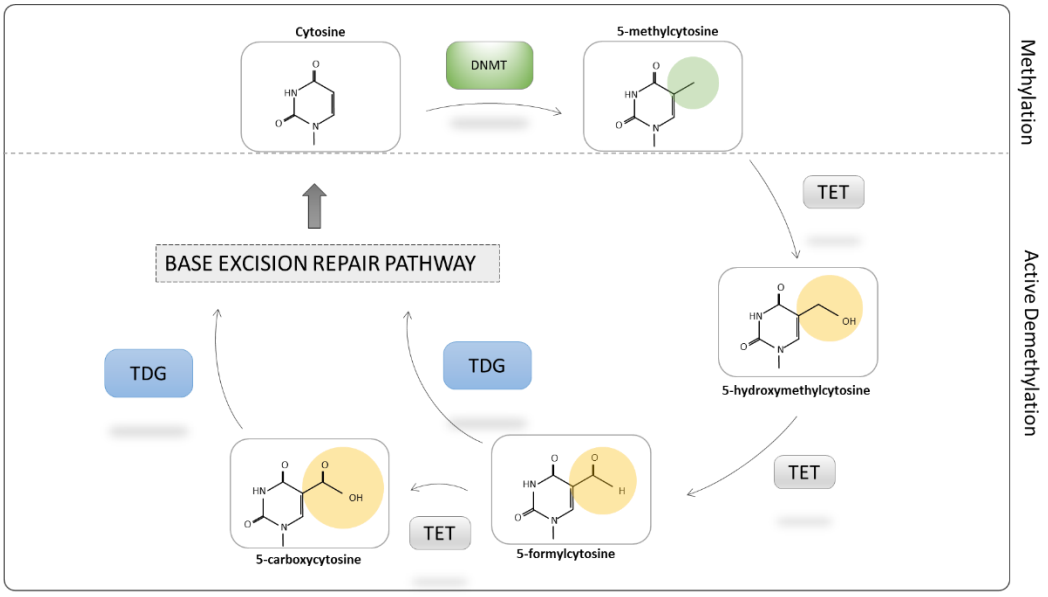
wide-spread occurring in brain as well as skeletal muscle and has been reported to occur in response to extracellular signals such as TGFβ, retinoic acid, and fibroblast growth factor 1 (FGF1) (Bruniquel and Schwartz, 2003; Guo et al., 2011; Zhang et al., 2007).

While it was originally hypothesized that active demethylation occurs through direct enzymatic removal of the methyl group from cytosine (as is observed in certain plant species), no enzyme capable of this excision have been identified in human cells. Instead, in mammalian systems, the removal of 5mC is dependent on a step-wise oxidation process, catalyzed by members of Ten Eleven Translocation (TET) family of proteins. This iterative oxidation process starts at 5mC and first generates 5-Hydroxymethylcytosine (5hmC) which is then further oxidized by TET proteins to 5-Formylcytosine(5fC) and finally to 5-Carboxylcytosine(5caC) (Ito et al., 2011). 5fC and 5caC are then cleaved by TDG which, in a highly coordinate series of steps involving APE1 and members of the Base Excision Repair pathway, re-establishes an unmodified cytosine at the site (Maiti and Drohat, 2011) (figure 1-4).

Active demethylation has been primarily investigated in the context of promoters, where TDG-dependent demethylation was required for expression of gene-target(s). However, recent work in embryonic stem cells has found that TDG depletion results in the accumulation of 5mC metabolites at distal enhancers (Song et al., 2013; Wheldon et al., 2014). While methylation status of promoters strongly correlates with gene expression, an analysis of 390 ER-positive breast tumors revealed that methylation of distal sites corelates

Figure 1-4. Active demethylation pathway.

Recent studies have established that TET-mediated, iterative oxidation, followed by TDG excision and BER is likely the most prevalent method through which active demethylation is achieved in mammals.


Cytosine
DNMT
5-methylcytosine
Methylation
TET
BASE EXCISION REPAIR PATHWAY
5-hydroxymethylcytosine
TDG
TDG
TET
TET
5-carboxycytosine
5-formylcytosine
Active Demethylation

to target gene expression more closely than promoter methylation. The significance of this remains to be elucidated and the exact role that TDG-mediated active demethylation plays at enhancers in this context, remains unresolved.

1.2.5 Enhancers and enhancer RNA (eRNA)

In addition to proximal regulatory elements, regulatory regions can also be found large distances away (up to millions of bases away or on different chromosome altogether) from specific genes. These regulatory regions, called 'enhancers', are able to mediate transcriptional activity of associated gene(s) (Jeong et al., 2014; Li et al., 2013; Ong and Corces, 2012; Yip et al., 2012).

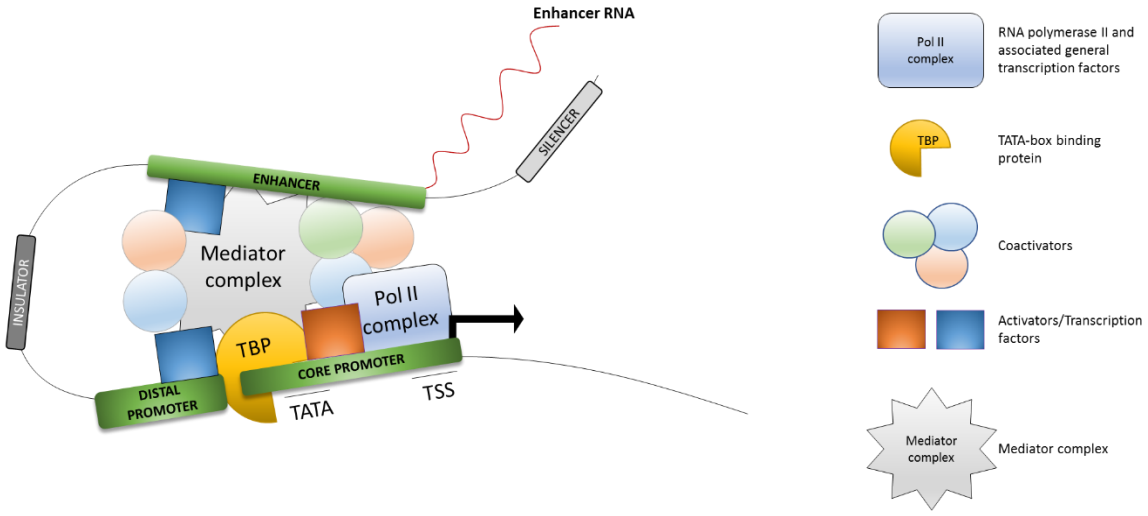
Functionally, enhancers share some similarities with promoters and often contain binding sites for transcription factors, RNA Pol II and components of the pre-initiation complex as well as coregulators (Plank and Dean, 2014). Enhancers are often located in non-coding regions of DNA and, in response to an activating signal, some enhancers are transcribed to produce long non-coding RNA, termed 'enhancer RNA' (eRNA) (Lam et al., 2014; Li et al., 2013; Pulakanti et al., 2013). While the role of eRNA still remains somewhat controversial, recent studies have confirmed that eRNA production, *per se*, causes conformational changes that bring the enhancer in direct contact with the target-gene promoter (Li et al., 2013). This brings together transcription-factors, coregulators and RNA-Pol II bound at the enhancer and the basal transcriptional machinery positioned at the proximal promoter (figure 1-5). Disruption of either eRNA production or looping can prevent transcription of the associated gene (Li et al., 2013).

While conventional reporter/luciferase assays are still used to identify potential enhancers, recent developments in high-throughput sequencing have allowed us to gain unprecedented insight into their structure and function. Poised or active enhancers are often found in 'open' chromatin regions depleted of nucleosomes - allowing for transcription factor/coregulator accessibility (Pulakanti et al., 2013). DNase-Seq and FAIRE-Seq are both techniques which can identify nucleosome depleted regions by digesting accessible DNA using DNase I or by depleting the histone-associated 'closed' regions, respectively. Unfortunately, these techniques alone provide an incomplete picture, as not all open regions are necessarily enhancers, and not all enhancers will necessarily be found in an 'open' chromatin state.

As identified by conventional biochemical techniques, enhancers are often sites of transcription factor and coactivator binding (Frietze et al., 2012). This property has been used to predict/identify potential enhancers by searching for genomic regions outside of annotated gene's that contain a cluster of transcription binding motifs or that contain a sequence conserved between species. However, the location of clustered transcription factor binding motifs or highly conserved sequence, does not necessarily mean that the region is an enhancer, as many transcription factor binding motifs are not actually bound by the transcription factor *in vivo* or are only bound by those TF's in certain cells, or in certain contexts. A more accurate way to identify/predict enhancer regions is through the identification of certain coregulator binding sites (Shlyueva et al., 2014).

Figure 1-5. Schematic of Enhancer and Promoter in 'active' configuration.

An example of a potential enhancer/promoter complex formed prior to release of Pol II and transcription. The composition of components at enhancers differs depending on context, however certain elements (Activators, coactivators, RNA Pol II, etc.) appear to be required.


Enhancer RNA
SILENCER
ENHANCER
INSULATOR
Mediator complex
Pol II complex
TBP
CORE PROMOTER
DISTAL PROMOTER
TATA
TSS
Pol II complex
RNA polymerase II and associated general transcription factors
TBP
TATA-box binding protein
Coactivators
Activators/Transcription factors
Mediator complex
Mediator complex

Because coregulators do not bind DNA directly (rather they are largely recruited by their interaction with specific transcription factors), global ChIP-Seq targeting cofactors such as the lysine acetyltransferase CBP (or its homologue p300) would identify regions that are bound by both CBP/p300 and their interacting transcription factor, removing the noise from the cruder motif analysis or sequence conservation studies. The binding of specific coactivators or corepressors to enhancers often results in histone markings that indicate the 'state' of the enhancer. For example, acetylation of histone H3 on Lysine 27 (H3K27Ac) is often found at enhancers and promoters that are actively being transcribed while H3K4me1, on the other hand, is a mark that is often found at enhancers but not at promoters (Bulger and Groudine, 2011; Lupien et al., 2008). Regions that contain both H3K27Ac and H3K4me1, have been strongly associated with actively transcribed enhancers (Creyghton et al., 2010; Zentner et al., 2011). Additionally, histone marks indicating repressed states also exist. For example, H3K27me3 (Histone H3, Lysine residue 27, tri-methylation) is often associated with transcriptional silencing and corresponds to the presence of the Polycomb complex, a protein complex that mediates silencing of target regions (Zentner et al., 2011). Collectively, the specific set of histone marks at an enhancer are often able to accurately characterize its state (i.e. "poised", "silenced" and "active") and are now widely used throughout enhancer related studies (Shlyueva et al., 2014).

Because enhancers are often transcribed into eRNA, which have been shown to play functional roles in 3-dimensional re-organization, techniques that are able to directly assay for these features are extremely powerful ways to identify or predict enhancers. Global Run-On Sequencing (GRO-Seq) is an accurate way to identify regions of the genome where RNA Pol II is actively engaged in transcription and has been used to identify

enhancers which produce eRNA in response to stimuli (Li et al., 2013). Various techniques exist which can be used to identify chromosomal conformation such as Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET). ChIA-PET allows for the capture of 3-dimensional interactions that occur at sites of a protein of interest. As enhancers are often characterized by interaction with the promoter and transcription (of both eRNA from the enhancer and mRNA from the gene-target), ChIA-PET performed with RNA Pol II has been used to successfully identify regions which are bonafide enhancers partaking in promoter interactions (Fullwood et al., 2009; Li et al., 2012). Alone, or in combination, these techniques have allowed for the accurate identification and mapping of enhancers globally.

Enhancers play a fundamental role in cell-specific transcriptional regulation and the importance of enhancers in human biology is wide-spread. During embryonic development for example, where precise patterns of gene expression are required, enhancers play a fundamental role in controlling the spatial and temporal transcription of key modulators of embryonic development (Creyghton et al., 2010; Ong and Corces, 2012; Zhu et al., 2012). Errors in enhancer sequence can lead to a predisposition to many common diseases, including cancer. Global methylation studies examining the association between methylation and gene dysregulation in cancer in fact found that the transcript level of genes more strongly correlated to its enhancer methylation status than to the methylation status of its promoter (Aran and Hellman, 2013; Wiench et al., 2011). A deeper understanding of enhancer regulation may be useful for treatment of pathologies that are underpinned by a dysfunctional regulation program.

1.3 Thymine DNA Glycosylase (TDG)

TDG was originally discovered by protein purification of factors capable of binding and processing T:G mismatches in HeLa cells (Neddermann and Jiricny, 1993). TDG is, a member of the "Mismatch Uracil Glycosylase" (MUG) branch of the "Monofunctional Uracil-DNA Glycosylase" (UDG) superfamily. Members of this family are characterized by their ability to excise mismatched Uracils (G/T or G/U mispairs) from double-stranded DNA. Structurally, TDG is composed of a catalytic 'core' flanked by N- and C- terminals. All three of its domains contain binding sites that mediate its physical interaction with a diverse set of proteins including components of the Base Excision Repair (BER), Nuclear Receptors (NRs), as well as additional coregulators (Figure 1-6). Additionally, both the N- and C- terminals are targets of post-translational modifications that have been shown to alter the localization and activity of TDG (Hardeland et al., 2000; Hashimoto et al., 2013).

1.3.1 TDG - Glycosylase Activity and Function

While originally recognized as an enzyme capable of cleaving Thymine from T:G mismatches, subsequent work has revealed that TDG is capable of accommodating and processing a much broader set of substrates found at the cytosine position in a cytosine:guanine (CpG) context (Cortázar et al., 2007).

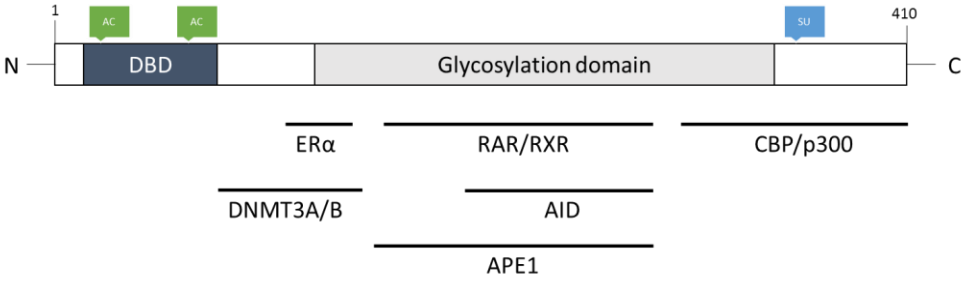
The crystal structure has revealed that TDG's catalytic domain forms a large hydrophobic pocket capable of accommodating various adducts and derivatives of uracil and cytosine including products resulting from oxidation, halogenation and deamination of these bases

and importantly, in the case of active demethylation, allows for the accommodation of the 5mC derivatives 5fC and 5caC(Maiti and Drohat, 2011; Raiber et al., 2012; Shen et al., 2013).

The finding that TDG interacts with many transcription factors and coregulators suggests that it can be targeted to specific sites through interaction with its binding partners (Cortázar et al., 2007). Once localized to a site targeted for demethylation, TDG forms specific interactions with the base being targeted for removal, a guanine adjacent to the target site and importantly, the guanine opposite the base target for removal (Hashimoto et al., 2013). The interactions between TDG and both guanines is of particular importance as it allows for a control of specificity: interaction with the adjacent guanine confirms that TDG is at a CpG site and interaction with guanine from the opposite strand confirms that TDG is bound to double stranded DNA (Cortázar et al., 2007). These and other interactions stabilize TDG at the targeted site and result in TDG intercalating with the DNA in place of the targeted base, which is instead 'flipped' out of the double-helix and into the catalytic site of TDG, ultimately leading to its cleavage (Hashimoto et al., 2013). Evidence for a 2nd TDG molecule being present and taking part in the interactions has also been shown (Cortázar et al., 2007). Cleavage of the mispaired base by TDG creates an abasic site which is 'nicked' by Apurinic/apyrimidinic endonuclease (APE1). This process is highly coordinated – once TDG cleaves the mispaired base, it notably binds the abasic site with a high affinity.

Figure 1-6. Schematic of TDG Protein.

Proteins known to interact with TDG are shown at approximate location where the interaction is believed to take place. Regions known to be post translationally modified in certain contexts are marked: Ac - Acetylation, S – Sumoylation**.**


1
AC
AC
SU
410
N
DBD
Glycosylation domain
C
ERα
RAR/RXR
CBP/p300
DNMT3A/B
AID
APE1

In order to ensure that the components required to carry out the next series of steps are present, TDG dissociation from the abasic site requires both APE1 binding and SUMOylation of TDG (Waters et al., 1999). The site is then repaired by DNA Polymerase β and DNA ligase restoring an unmodified Cytosine at the site of repair.

1.3.2 TDG as a Coactivator

Evidence that TDG possesses functions independent of its glycosylase activity was first demonstrated in studies investigating the role of TDG in CBP/p300-dependent transcription (Tini et al., 2002). Using a combination of immunoprecipitation, reporter and Avidin-biotin-coupled DNA assays, researchers demonstrated that TDG recruits and forms a complex with CBP/p300, stimulating its transcriptional activity (Tini et al., 2002). Importantly, inactivation of TDG's glycosylase activity using a point mutation did not affect its ability to bind or stimulate CBP/p300-dependent transcription, suggesting that TDG may be behaving primarily as a coactivator.

Further evidence for the coactivator role for TDG has emerged from work in Estrogen receptor signaling. ERα, a member of the nuclear receptor family that is responsible for estrogen responsiveness in mammals and activation of ERα signaling has been implicated in breast cancer progression. It has been shown that TDG forms a complex with the ERα in response to ligand and localizes to ER-target promoters. Reporter assays directly testing the role of TDG in E2-mediated signaling revealed that TDG is a critical mediator of ER-signaling and deletion of TDG inhibits transcription of ER-target genes. Importantly, a

catalytically inactive mutant had no effect on TDG's ability to mediate E2-transcription, further supporting its a role primarily as a coactivator (Chen et al., 2003).

Similar observations have been made regarding TDG's role in Wnt signaling. Wnt signaling underlies many fundamental biological processes during embryogenesis and importantly, is often dysregulated in cancers. In colorectal cancer (CRC) aberrant Wnt signaling drives proliferation and progression of the disease (Xu et al., 2014). TDG forms a complex with CBP and TCF4, a transcription factor critical to Wnt signaling that localizes to Wnt-targets and potentiates Wnt-signaling. ChIP assays at c-myc, in conjunction with shRNA-mediated depletion of TDG, revealed that TDG binding is concomitant with H3 acetylation (a function of CBP/p300) at the site which was not observed upon TDG depletion (Xu et al., 2014). These findings suggest that TDG facilitates transcriptionally favorable alterations to the chromatin environment through its recruitment of CBP/p300 to Wnt targets. Similar to previous reports, a catalytically inactive mutant of TDG had no effects on TDG's ability to drive Wnt signaling, again supporting the notion that TDG's functionality is primarily that of a scaffold in certain contexts.

Further work characterizing the functional role of TDG has found using mouse knockout models, where TDG knockout is embryonic lethal (Cortázar et al., 2011; Cortellino et al., 2011). Interestingly, one group found that Tdg knockout mice exhibited phenotypes that resembled both that of CBP/p300 null mice embryos as well as those deficient in select RAR/RXR genes, suggesting that TDG deletion may disrupt CBP/p300-mediated and/or retinoic acid signaling critical to proper development. TDG null mice have severe defects in both CBP/p300 and RAR/RXR-dependent transcriptional activity. Furthermore, it was shown that TDG is responsible for mediating the interaction between RAR/RXR and

CBP/p300 and that deletion of TDG prevented CBP/p300 recruitment to RAR/RXR targeted genes and also resulted in a loss of H3 acetylation(Cortellino et al., 2011; Hassan et al., 2017). Work by a separate group found that in addition to disrupting CBP/p300 binding at known RAR/RXR sites, TDG deletion disrupted CBP/p300 recruitment at various other loci important for development. In addition to disruption of CBP/p300 recruitment, TDG deletion disrupted recruitment of other important factors including GADD45a, AID and MLL1(Cortázar et al., 2011).

Interestingly, disrupting the glycosylase activity of TDG in mouse models is also embryonic lethal (Cortellino et al., 2011). While it is not entirely clear why this occurs, TDG's glycosylase activity has been previously shown to be important for mediating RAR/RXR-mediated transcription. RAR/RXR target genes have been shown to have methylated promoters to which TDG is recruited, suggesting that in cases whether active demethylation of a promoter is required for gene specific transcription, TDG's glycosylase activity becomes essential.

Finally recent studies have shown that TDG is important for maintaining enhancers in a hypomethylated state in embryonic stem cells (Raiber et al., 2012; Wheldon et al., 2014). This is important as enhancers can be transcribed to produce long non-coding RNA and importantly, these enhancer RNA's can have functional effects on gene-targets, sometimes hundreds of thousands of base pairs away. While TDG's role in transcriptional activation is well established at promoters, whether or not TDG plays a role in the transcription of eRNA at enhancers has not yet been investigated. Taken together, TDG's role as a coactivator plays a central role in its ability to mediate a diverse set of signaling pathways, however the mechanism(s) remain unclear.

1.4 Rationale

To expand the body of knowledge surrounding TDG I explored its role in two systems: ERα mediated signaling in the context of the MCF7 breast cancer line, and cellular senescence in the context of healthy tissue.

The steroid hormone receptor ERα is overexpressed in many breast cancers and is often the target of endocrine therapies, such as tamoxifen. Importantly, TDG interacts with ERα and potentiates its action in a ligand-specific manner (Chen et al., 2003). In the classical mechanism of ERα function, ERα is found mainly in the cytosol and binding of ligand causes a conformational change that is concomitant with ERα dimer formation and translocation into the nucleus. Activated ERα can bind DNA directly through its interaction with ERE's, or the binding can be indirect and mediated through an interaction with various transcription factors. Work focusing specifically on the TFF1/PS2 gene promoter, a well-studied target of ER-mediated signaling, has found that, in addition to the recruitment of ER, E2 treatment resulted in the recruitment of large number of proteins to the TFF1 promoter that includes histone acetyl transferases and histone methyl transferases, the nucleosome remodeling complex SWI/SNF and general transcription factors (Métivier et al., 2003, 2008). Experiments which examined the kinetics of cofactor binding revealed that rather than binding simultaneously, the recruitment and release of coactivators was both combinatorial and cyclical in nature, with certain "sets" of factors binding while others released, repeatedly (Métivier et al., 2003). In addition to the previously mentioned factors, the promoter of TFF1 underwent cyclical methylation/demethylation during transcriptional activation. Importantly, the increase in methylation coincided with an increased binding of DNMT1 while demethylation coincided with release of DNMT1 and

binding of TDG. siRNA mediated depletion of TDG resulted in an inability to effectively remove the methylation and prevented TFF1 transcription (Métivier et al., 2008). The cyclical methylation and demethylation is concomitant with binding of various cofactors and chromatin remodelers, including TDG. Remarkably, knockdown of TDG using siRNA resulted in a hypermethylation of the promoter and the inability to induce TFF1 using E2, highlighting its critical importance in ERα signaling.

Recent high-throughput studies have provided a more global picture of E2-dependent ERα binding. One surprising result from these studies was that a substantial portion of ERα binding occurred at distal regulatory regions containing histone modifications that mark enhancer sites (H3K27ac and H3K4me1). Studies employing GRO-Seq, which identifies nascent transcription genome-wide, found that ERα was localized to the enhancers of approximately ~90% of genes that showed upregulation in response to E2 (in contrast to only ~9% that showed binding of ERα at their promoters) (Li et al., 2013).

While enhancers are often located in non-coding regions, in response to E2 the majority (~83%) of enhancers associated with E2-regulated genes are transcribed. At the time it was unclear whether transcription of eRNA was transcriptional noise due to the presence of RNA Pol II or played a functional role. However recent studies have shown that eRNAs *per se* may be required for target-gene transcription in different contexts (Hsieh et al., 2014; Li et al., 2013; Melo et al., 2013). ChIA-PET is a technique capable of identifying long-distance chromatin interactions. These studies revealed that in response to E2, chromatin rearrangement surrounding ER-dependent genes occurred by bringing into proximity target-gene promoters with enhancers, which often times contain transcription factors, coregulators and RNA Pol II, potentiating transcription of the target gene. Importantly,

eRNA production at enhancers was shown to mediate this looping (Li et al., 2013). While the mechanism through which eRNA regulates 3-dimensional re-organization is still unclear, eRNA has been shown to directly recruit chromatin coactivators that may take part in promoter-enhancer complex formation (Bose and Berger, 2017).

Generally, eRNA production often occurs at enhancers that are TET occupied and hypomethylated (Pulakanti et al., 2013). This is interesting, as recent work in mouse embryonic stem cells has shown that TDG localizes to enhancers and functions to maintain them in a hypomethylated state. Furthermore, eRNA production drives 3-dimensional reorganization that mediates ER-dependent transcription and I have recently found that TDG mediates similar 3-dimensional reorganization during RAR signaling (Hassan et al., 2017). While TDG's ability to regulate ERα signaling has been established through reporter assays as well through targeted assays at specific genes (i.e. TFF1), its role globally remains to be explored.

The second component of my research focuses on the role of TDG in cellular senescence. Senescence is a state of persistence cell-cycle arrest that occurs when cells have exceeded their proliferative capacity. Proliferative senescence is a complex damage response mechanism resulting from telomere depletion and causes an upregulation of cell-cycle inhibitors and down regulation of pro cell-cycle signals(Rayess et al., 2012). In addition to exceeding proliferative capacity, cells can be induced to senesce by exposure to ultra-violet radiation, oxidative stress as well as oncogene expression. The senescence program is a critical barrier to cancer. Senescing cells have distinctive features that distinguish them from their healthy counterparts. Most obvious is a distinct, flattened, morphology and an enlarged cytoplasm. These cells will stain blue when exposed to X-gal under acidic

conditions due to an enlarged lysosome cavity and abundance of beta-galactosidase enzyme (Kurz et al., 2000). Transcriptionally, senescing cells often overexpress the tumor suppressors p53, p21, ARF and CDKN2A (Chen et al., 1998a).

CDKN2A and ARF are potent tumor suppressors that are found at the INK4 locus (in conjunction with a third tumor suppressor CDKN2B and the long non-coding RNA, ANRIL). Importantly, the transcription of gene-products at this locus sensitive to epigenetic regulation: Specifically, methylation of their promoters. In many cancers the promoters of these genes are hypermethylated, and demethylation causes re-expression. For example, CDKN2B transcription, which is silenced through promoter methylation, can be activated by TGFβ which, in certain contexts, removes promoter methylation (Thillainadesan et al., 2012). Importantly, TDG has been shown to be a critical component of the active demethylation process at this promoter, and loss of TDG results ablates TGFβ's ability to induce demethylation of the CDKN2B promoter and its transcription. Similarly, reporter assays have revealed that when CDKN2A is hypermethylated, TDG is required for its active demethylation and expression(Hu et al., 2010).

1.5 Objectives

To investigate the mechanism through which TDG regulates transcription, I treated the MCF-7 breast cancer cell line with E2, an ERα agonist, and performed ChIP-Seq using an antibody specific for TDG to map the locations to which it localizes (Chapter 2). Bioinformatic analysis was performed to characterize (A) the binding sites of TDG genome wide relative to genomic annotation (B) the overlap between ERα and TDG (C) the overlap

between known transcription factors from the ENCODE database and (C) the histone marks at these sites. Based on this analysis I hypothesize that in response to E2 TDG regulates ER-target gene transcription through its recruitment to enhancers where it mediates the transcription of eRNA and 3-dimensional organization. To test the hypothesis, I performed siRNA mediated knockdown experiments in conjunction with real-time qPCR at both target-gene mRNA levels and enhancer eRNA levels from the same set of ER-target genes. Chromosome Conformation Capture (3C) was performed at GREB1, a well-studied ERα target gene, to determine whether TDG impacted 3-dimensional organization. Finally, using both siRNA-depletion and CRISPR-knockout cells, I investigated and characterized the role of TDG in proliferation, adhesion, migration and invasion in the context of MCF-7 breast cancer cells.

In chapter 3, I investigate the role of TDG in senescence in the context of development and in human adult fibroblasts. To determine the role of TDG in senescence I depleted human lung fibroblasts of TDG using siRNA and treated them with sublethal, senescence-inducing, levels of hydrogen peroxide and monitored proliferation using growth curves and CDKN2A induction using western blotting. To delineate a potential mechanism through which TDG regulates CDKN2A, I interrogated the methylation status of the CDKN2A promoter and performed ChIP using an antibody specific to CBP in the presence and absence of H_2O_2.

TDG knockout is embryonic lethal, with mice dying at approximately E11.5. Interestingly, recent work has revealed that during development in mice a highly regulated senescence program is initiated, and specific structures being undergoing senescence around the time knockout TDG mice die. As no previous study has been published examining the role of

TDG in developmental senescence, I generated TDG knockout embryo's and performed beta-galactosidase staining and conducted a double-blind study to determine whether TDG is important for execution of the embryonic senescence program in mice. Finally, to identify genes affected by TDG knockout I performed RNA-Seq and bioinformatic analysis on wildtype and TDG KO embryos.

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Chapter 2. Genome-wide Analysis Reveals a Role for TDG in Estrogen Receptor-mediated Enhancer RNA Transcription and 3-Dimensional Reorganization.

2.1 Introduction

Steroid hormones such as 17β-estradiol (E2) coordinate complex gene programs and exert profound effects on cell growth, development and homeostasis(Farooq, 2015). E2 mediates its biological effects by binding to, and activating, the estrogen receptor (ERα and ERβ). The ERs are members of the nuclear hormone receptor superfamily which function as ligand-activated transcription factors. In the classical mechanism of hormone action, E2 binding induces receptor dimerization which facilitates binding to genomic DNA at specific sequences in the regulatory region of ERα responsive genes called "estrogen response elements". Importantly, ligand-bound ERα undergoes a conformational change that facilitates the recruitment of coactivator proteins that coordinate specific transcriptional responses.

Genome-wide studies using ChIP-based technologies have shown that the majority of ERα binding sites in breast cancer cells are found distally from gene promoters, and a significant component are found within gene-specific "enhancer" regions in response to E2 (Jin et al., 2015). Enhancers are essential regulatory regions found in non-coding regions that control temporal and tissue-specific gene expression. Furthermore, given that less than 2% of the mammalian genome accounts for protein-coding genes, an increasing number of mutations and aberrant methylation patterns associated with breast cancer have been found to reside in enhancer regions (Aran and Hellman, 2013). In addition to recruiting specific transcription factors enhancers also bind specific coregulators and components of the transcriptional machinery, including RNA polymerase II. Importantly, some enhancers are actively transcribed into long non-coding RNAs known as enhancer RNAs (eRNAs)(Li et al., 2013). While the exact role of eRNAs remains controversial, some eRNAs have been

shown to regulate gene expression by causing a 3-dimensional conformational change bringing together the promoter, enhancer, and transcriptional machinery into 'transcriptional pockets' (Hsieh et al., 2014; Li et al., 2013). It has been shown that E2 rapidly increases eRNA production at many sites of ERα binding and results in the activation of adjacent genes (Li et al., 2013). Although the exact mechanism governing eRNA transcription is unclear, recent evidence suggests that enhancer methylation status may play a role in eRNA production (Pulakanti et al., 2013).

DNA methylation occurs at the C5 position of cytosine (5mC) and is found primarily in a cytosine-guanine (CpG) context. Genome wide patterns of CpG methylation are deposited by the DNA methyltransferases (DNMTs) and are important for the establishment of proper chromatin states that are associated with normal development and cellular homeostasis (Smith and Meissner, 2013). 5mCs function as targets for methyl-binding domain proteins which can subsequently recruit additional chromatin remodelers and co-repressors (Moore et al., 2013). Furthermore, in a promoter context, methylated CpGs can render the site inaccessible to the transcriptional machinery resulting in transcriptional silencing. Interestingly, while the majority of genomic DNA is methylated at CpGs (Ehrlich et al., 1982), 40-70% of gene promoters contain long stretches of CpG clusters (CpG islands) that are unmethylated based on bisulphite sequencing analysis (Saxonov et al., 2006). This pattern of global hyper-methylation and promoter hypo-methylation is present in healthy tissue and in differentiated cell types. Importantly, improper control of the setting and erasure of these marks has been implicated in various pathological phenotypes, such as cancer and abnormal embryogenesis (Baylin and Herman, 2000; Haaf, 2006).

Whereas the mechanism of DNA methylation is well understood, a unifying mechanism for DNA demethylation has not been unequivocally identified. DNA demethylation may occur passively when newly synthesized DNA strands remain unmethylated during successive rounds of DNA replication, as a result of DNMT1 inhibition. In contrast, active demethylation is a replication-independent process involving the DNA glycosylase Thymine DNA glycosylase (TDG). In one scenario, cytidine deaminases such as activation-induced deaminase (AID) or Apolipoprotein B mRNA editing enzymes (APOBEC 1-4) convert 5mC to thymine, generating a G:T mispair (Suspène et al., 2005). Excision of mispaired thymine by TDG initiates the base excision repair pathway (BER) which effectively restores unmethylated cytosine. However, this model has been challenged recently because AID/APOBEC members are much less active on 5mC and its derivatives *in vitro* and *in vivo* (Nabel et al., 2012). A more plausible mechanism involves the Ten Eleven Translocation (TET 1-3) enzymes which oxidize 5mC to 5-hydroxymethylcytosine (5hmC). Subsequently, 5hmC is then metabolized further by TETs into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito et al., 2011). These oxidized 5mC metabolites, 5fC and 5caC, are recognized and removed by TDG (Hashimoto et al., 2013; Maiti and Drohat, 2011). In addition, an alternative mechanism has been postulated for ER-dependent demethylation in breast cancer cells(Periyasamy et al., 2015). ER-dependent transcriptional activation at the TFF1 promoter requires cyclic patterns of methylation and demethylation, that is mediated by recruitment of TDG in concert with DNMT 3a and 3b (Métivier et al., 2003, 2008). It has been postulated that Dnmt3a/b in addition to catalyzing *de novo* DNA methylation, can facilitate demethylation by deaminating 5mC when SAM levels are limiting (van der Wijst et al., 2015).

The generation of TDG knockout mice has corroborated the importance of TDG in regulating active demethylation and tissue specific gene expression. Deletion of TDG in the germline is embryonic lethal and leads to DNA hypermethylation and defects in the expression of various developmentally regulated genes (Cortázar et al., 2011; Cortellino et al., 2011) Additionally, 5fC and 5caC levels increase five to ten fold genome wide in TDG null ES cells (Wu et al., 2014), (Song et al., 2013). TDG has also been implicated in transcriptional control and gene expression by functioning as a molecular scaffold protein. TDG interacts directly with ERα in a ligand dependent manner and co-localizes to the promoter of TFF1 (Chen et al., 2003) resulting in increased gene expression, effects which are lost when TDG is depleted (Métivier et al., 2008). TDG also interacts directly with other transcription factors and coregulators and in TDG null MEFs, the presence of TDG is required for recruiting the acetyltransferases CBP/p300, TET2 and other histone modifying enzymes to a subset of target genes (Cortázar et al., 2011; Cortellino et al., 2011; Hassan et al., 2017; Tini et al., 2002). These findings are consistent with the notion that TDG plays a central role in epigenetic stability and methylation control.

In this study, I generated a global profile of TDG binding in MCF7 breast cancer cells in response to E2 treatment using ChIP-Seq. I have integrated the data from our ChIP-Seq assays with data from other genomic assays to provide a global view of TDG binding. In response to E2 treatment, I show that TDG binds primarily to genomic regions upstream of target genes which, in addition to recruiting ERα and RNA Polymerase II, also bind various transcription factors, co-regulators and epigenetic modifiers including p300, GATA3 and Tcf7l2 and are marked by histone marks indicative of active enhancers. Importantly, TDG binds to regions which, in response to E2, transcribe eRNAs and take

part in 3-dimensional restructuring of the genome. Remarkably, at a subset of enhancers that E2 targets, I found that TDG depletion abrogates E2-mediated eRNA, disrupts 3-dimensional reorganization at ERα targets such as GREB1 and disrupts E2-mediated transcription of corresponding ER-target genes. To investigate whether TDG plays a functional role in E2 signaling in breast cancer, I engineered an MCF7 TDG-knockout cell line using CRISPR technology and found that TDG knockout and depletion leads to defects in E2 mediated proliferation and sensitizes MCF7 cells to the anti-estrogen, tamoxifen. Importantly, I also find that TDG depletion causes adhesion defects and drastically increases the migratory capacity and invasiveness of MCF7 cells. Collectively our findings suggest that TDG plays a central role in mediating the transcriptional and functional effects of E2 in breast cancer and may prove to be an effective therapeutic target.

2.2 Results

2.2.1 Global TDG binding in response to E_2

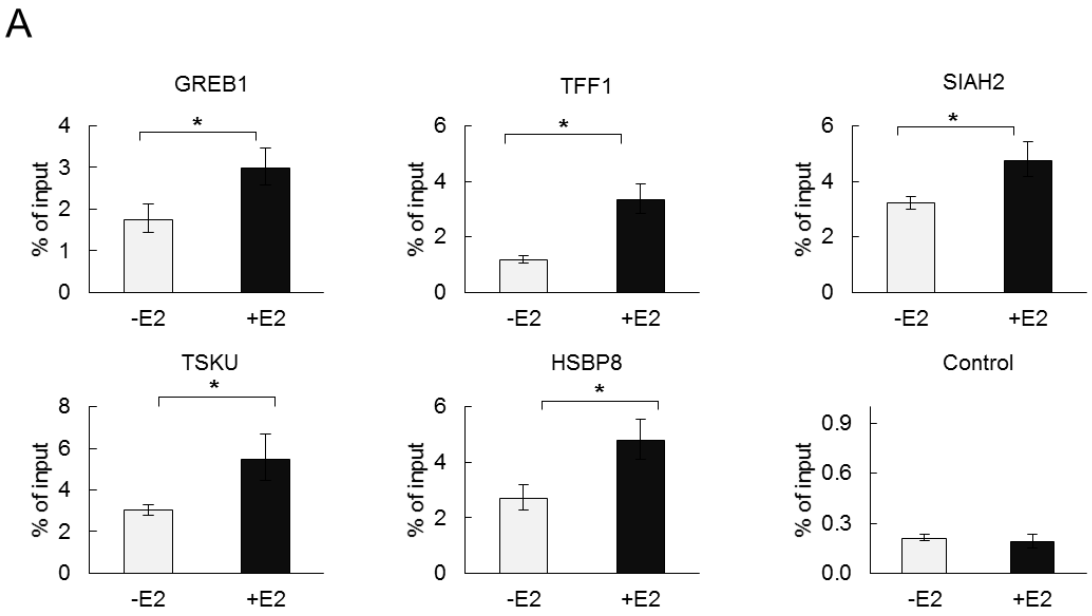
Previous reports have shown that, in response to E2, TDG physically interacts with ERα and localizes to TFF1/PS2, a well characterized ER-target gene(Métivier et al., 2008). To determine whether colocalization of TDG and ERα extends to other genomic locations, MCF7 cells were treated with 100 nM E2 for 45min and ChIP-Seq was performed using a TDG-specific antibody. Biological replicates were performed and for each replicate the reads were processed to remove duplicates and corrupted reads before being mapped to the human genome (hg19). Areas of significant enrichment were identified using an FDR 0.01. To identify only high confidence peaks, the peak set was filtered and only those peaks with

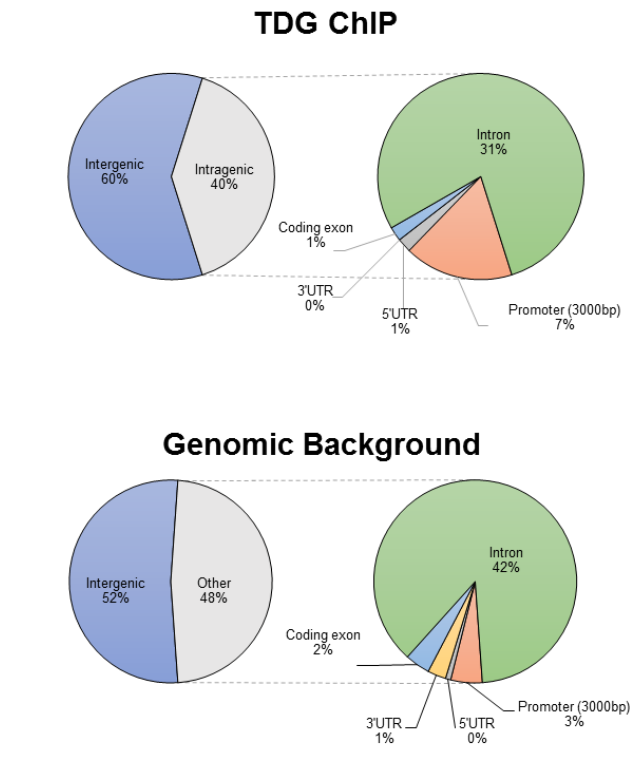
a p-value < 0.05 and fold-change greater than 1.2-fold were retained. Finally, by retaining only peaks which appeared in both biological replicates I was able to identify 117 highly-confident regions to which TDG localized in response to E2. Validations were performed using conventional ChIP-qPCR (Figure 2-1A). Compared to genomic background, global analysis of the high-confidence TDG peaks revealed that E2-dependent TDG binding was enriched at promoters (7% of total TDG peaks, compared with 3% genomic background) as well as distal to promoters with approximately 60% occurring intergenically (compared to 52% for background) (Figure 2-1 B). However, overlapping TDG peaks with sites of E2-dependent ERα localization revealed that 45% of TDG peaks occur at the same sites where ERα localizes in response to E2 (Figure 2-1 C and D).

Recent studies have shown that the ERα is found at enhancers and colocalizes with various transcription factors known to play important roles in enhancer regulation (Nguyen et al., 2014). The publicly available database, “Transcription Factor ChIP-Seq Uniform Peaks” from ENCODE contains the binding profiles of these and other transcription factors from numerous cell lines. I compared E2-dependent TDG binding to the 690 files available from ENCODE using two measures of similarity: Jaccard statistic and the Fisher exact test. Within the ENCODE datasets, those which most closely resemble E2-dependent TDG binding are the datasets from experiments recording ERα binding in response to E2

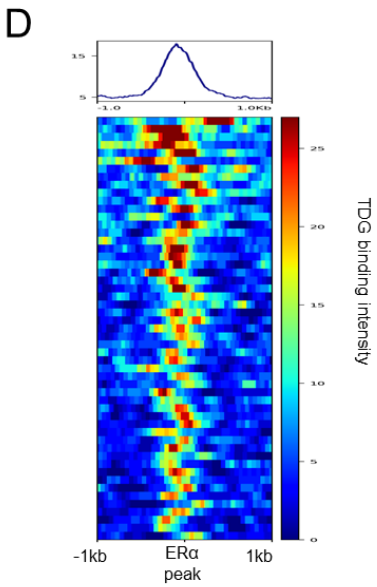
Figure 2-1. Global analysis of E2-dependent TDG localization.

(A) MCF7 cells were treated with 100nM of E2 (45min) and ChIP-qPCR was performed using TDG antibody. Region used as negative control shows low level of TDG binding in ChIP-Seq data with no change in levels after E2 treatment. (*p-value < 0.05, error bars represent standard deviation of the mean, n>2). (B) Sites of E2 dependent TDG binding were mapped to the annotated genome using CEAS (C) Venn diagram showing overlap between E2-dependent TDG peaks and ERα peaks obtained from public dataset. (D) Sites of ERα binding (-/+ 1000bp) overlaid with TDG binding signal, showing strong relationship between location of TDG binding and ERα binding at these regions.


A
GREB1
TFF1
SIAH2
TSKU
HSBP8
Control
% of input
-E2
+E2
*


B
TDG ChIP
Intergenic 60%
Intragenic 40%
Intron 31%
Coding exon 1%
3'UTR 0%
5'UTR 1%
Promoter (3000bp) 7%
Genomic Background
Intergenic 52%
Other 48%
Intron 42%
Coding exon 2%
3'UTR 1%
5'UTR 0%
Promoter (3000bp) 3%


C
ERalpha (10,196)
10,143
53
64
TDG (117)


D
-1kb
ERα peak
1kb
TDG binding intensity

treatments in breast cancer cells such as MCF7 cells and the metastatic T-47D breast cancer cell line. The other transcription factors that exhibit binding patterns most similar to that of TDG are p300, GATA3, Tcf7l2, the oncoprotein ZNF217, and RNA polymerase II (Figure 2-2A and B). Importantly, these proteins have been identified as having important roles at enhancers. Interestingly, I also observe higher similitude between TDG and both Myc and E2F1. While both proteins have been implicated in breast cancer progression, little is known concerning their respective roles at specific enhancers. Motif analysis focusing on TDG peaks that overlap with ERα revealed an enrichment for the canonical Estrogen Response Element motif, as well as the GATA DNA binding motif (Figure 2-2C). In contrast, TDG peaks that do not overlap with ERα are enriched for only a single motif, PU.1. Transcription factors from the GATA family regulate genes that are implicated in cell cycle arrest and cell survival (Zheng and Blobel, 2010). GATA3, specifically, has been identified as a critical component of mammary epithelial cells development and is 1 of 3 genes that have been shown to be mutated in >10% of breast cancers (Theodorou et al., 2013). Furthermore, GATA3 has been shown to mediate enhancer accessibility in MCF7 cells and its depletion results in an altered binding profile of ERα upon E2 treatment, with a corresponding altered change in target gene expression (Theodorou et al., 2013).

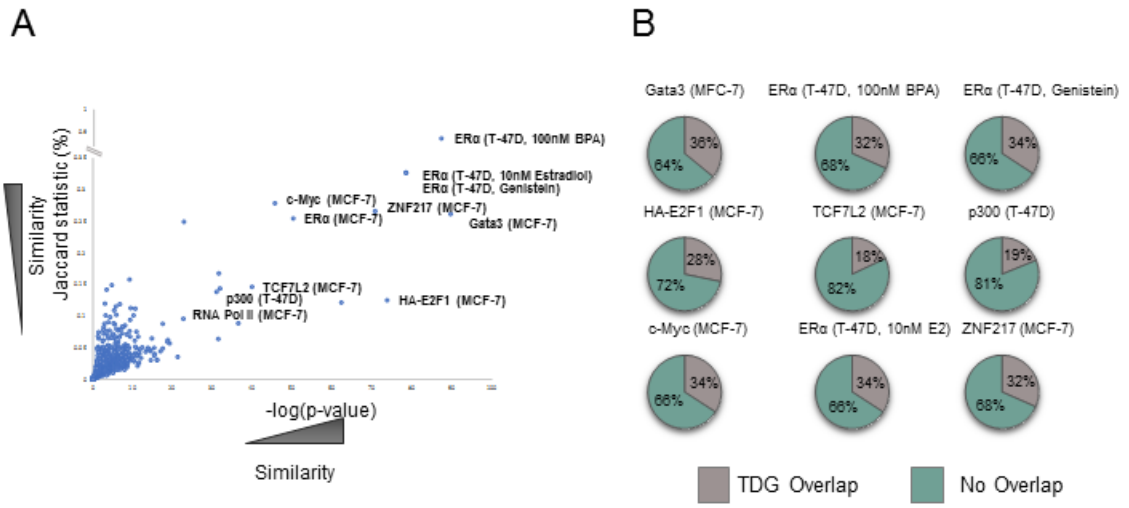
Distal regulatory sites involved in gene repression or activation are frequently marked by specific histone modifications. Enhancers involved in transcriptional activation high levels of H3K27Ac, H3K4me2 and H3K4me3 (Chen et al., 2015; Creyghton et al., 2010; Lupien et al., 2008) while sites involved in silencing are often devoid of most of these marks and

instead contain H3K9me3 and H3K27me3 (Zentner et al., 2011; Zhu et al., 2012). To gain a better understanding of the epigenetic makeup of sites to which TDG localizes in response to E2, I compared our data with that of publicly available ChIP-Seq datasets performed using antibodies against histone modifications. Aggregate plots and heatmaps at sites of TDG, or TDG and ER, localization in response to E2 revealed that TDG localizes with histones containing marks found at active enhancers (H3K27Ac, H3K4me2 and H3K4me3) and depleted almost entirely of histone markings corresponding to repressed or silenced enhancers (H3K9me3 and H3K27me3) (Figure 2-2D and E).

To determine the extent of overlap of TDG and the transcription factors identified in our global analysis, I cross-referenced genes whose transcription is induced upon E2 treatment with genes that are adjacent to TDG peaks (-/+ 100kb) to identify E2-inducible genes that are potentially regulated by TDG. A subset of genes meeting these criteria were selected and a closer examination of the genomic landscape surrounding sites of TDG was performed (Figure 2-3). Remarkably, I find highly-enriched binding occurs precisely at sites which bind the transcriptional factors identified in our original ENCODE analysis. Furthermore, these sites of E2-dependent TDG localization are enriched for histone marks that correspond to active enhancer while being devoid of marks corresponding to repressed/silenced enhancers, predicted by our previous bioinformatic analysis (Figure 2-2D and 2-2E). I also found a basal-level of TDG binding across the DNA at these regions which is likely reflective of TDG's non-specific DNA binding activity.

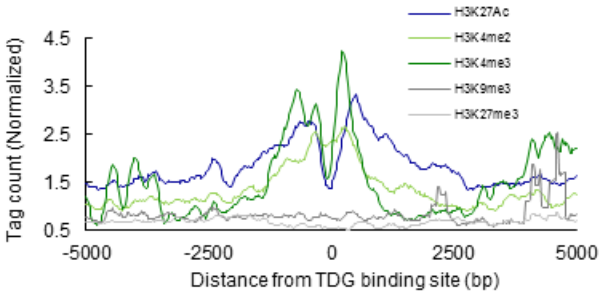
Figure 2-2. TDG localizes to sites occupied by transcription factors.

(A) Individual datasets obtained from the Transcription Factor ChIP-Seq Uniform Peaks dataset from ENCODE were compared to the TDG dataset using the Fisher exact test p-value and Jaccard statistic to determine relative similarity. A subset of the most similar matches is labelled with the cell type and treatment, if disclosed, in brackets (B) Overlap between TDG peaks and the corresponding ENCODE dataset (The average binding profile of the 10 least similar datasets from the ENCODE database was used as controls. None of the 10 least similar had more than a single peak which overlapped with our dataset). (C) Motif analysis performed on sites of E2 mediated TDG localization revealed the canonical estrogen response element (ERE) as a top hit followed by GATA protein consensus binding site. (D) Overlap of ChIP-Seq signal from publicly available histone datasets at sites of E2-dependent TDG binding in MCF7 cells. (E) Heatmaps showing intensity of histone marks at sites of TDG binding. Sites where TDG localizes in response to E2 are enriched for histone marks indicating 'active' enhancers (H3K27Ac, H3K4me2 and H3K4me3) while depleted for those marking 'repressed' enhancers (H3K9me3 and H3K27me3).



C

Rank	Motif	Name	p-value	% of target sequences with motif
1		ERE	$1*10^{-13}$	18.80%
2		GATA4	$1*10^{-4}$	21.37%
3		GATA1	$1*10^{-4}$	14.53%
4		GATA2	$1*10^{-4}$	15.38%
5		GATA3	$1*10^{-3}$	26.50%



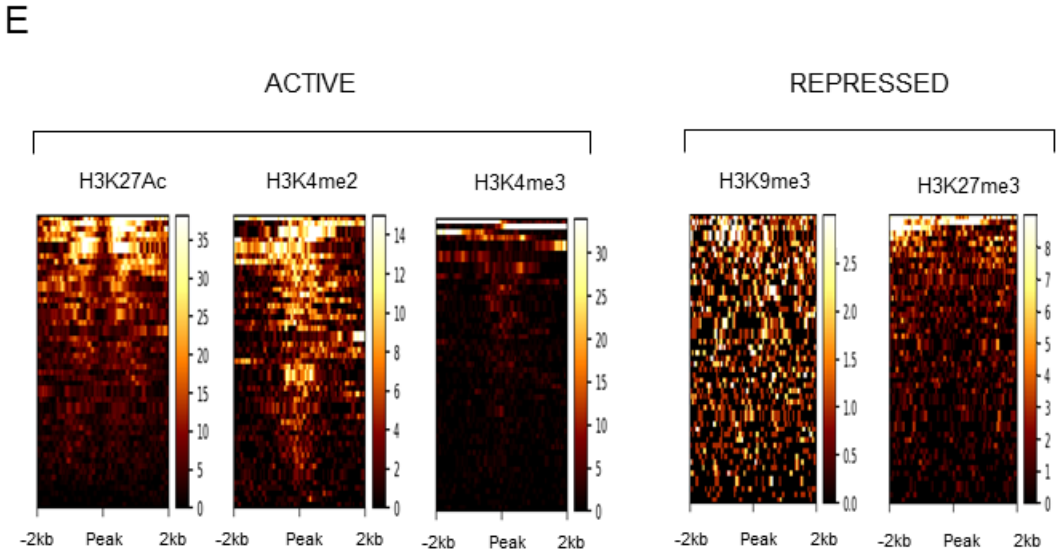
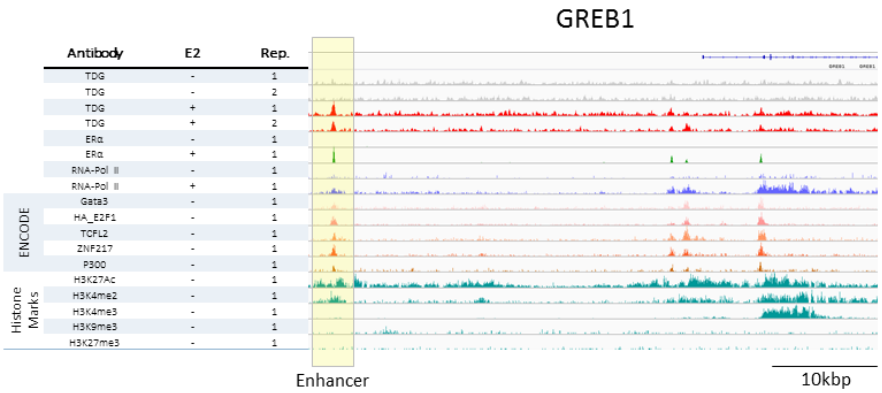
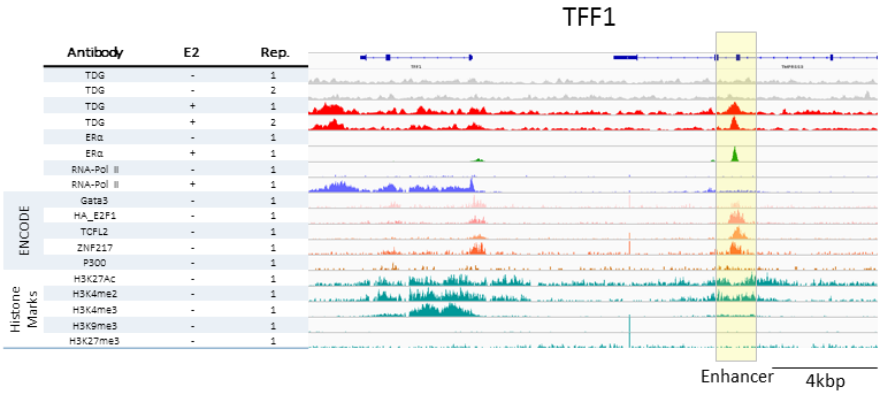
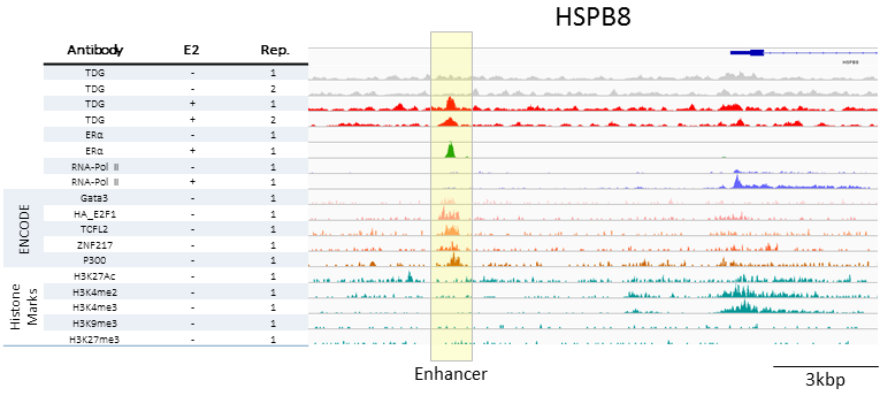


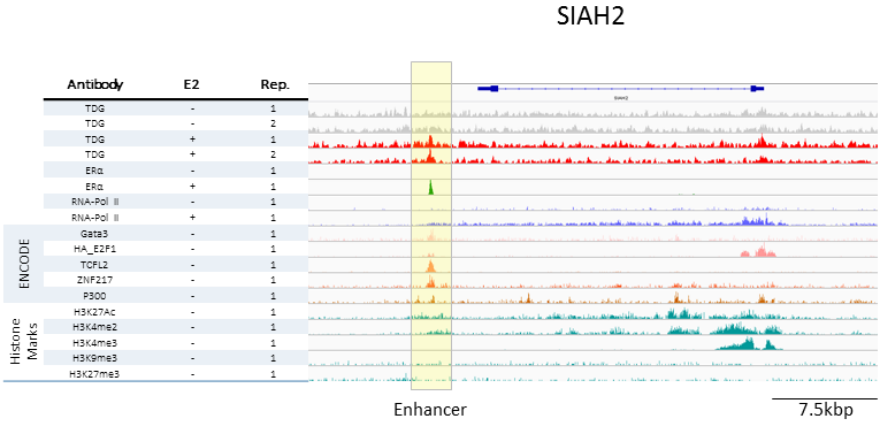
Figure 2-3. Transcription factor binding and histone modifications at a subset of TDG-targeted genes.

Genomic regions surrounding genes identified as having E2-dependent TDG binding and increased transcription show precise overlap of TDG, ERα and a subset of ENCODE transcription factors. Additionally, overlap with datasets containing histone ChIP-Seq data reveals the enrichment of histone marks corresponding to active/poised enhancers and depleted of those marking repressed/silenced enhancers (SM = standard media).


GREB1
Antibody E2 Rep.
TDG - 1
TDG - 2
TDG + 1
TDG + 2
ERα - 1
ERα + 1
RNA-Pol II - 1
RNA-Pol II + 1
Gata3 - 1
HA_E2F1 - 1
TCFL2 - 1
ZNF217 - 1
P300 - 1
H3K27Ac - 1
H3K4me2 - 1
H3K4me3 - 1
H3K9me3 - 1
H3K27me3 - 1
ENCODE
Histone Marks
Enhancer
10kbp


TFF1
Antibody E2 Rep.
TDG - 1
TDG - 2
TDG + 1
TDG + 2
ERα - 1
ERα + 1
RNA-Pol II - 1
RNA-Pol II + 1
Gata3 - 1
HA_E2F1 - 1
TCFL2 - 1
ZNF217 - 1
P300 - 1
H3K27Ac - 1
H3K4me2 - 1
H3K4me3 - 1
H3K9me3 - 1
H3K27me3 - 1
ENCODE
Histone Marks
Enhancer
4kbp


HSPB8
Antibody E2 Rep.
TDG - 1
TDG - 2
TDG + 1
TDG + 2
ERα - 1
ERα + 1
RNA-Pol II - 1
RNA-Pol II + 1
Gata3 - 1
HA_E2F1 - 1
TCFL2 - 1
ZNF217 - 1
P300 - 1
H3K27Ac - 1
H3K4me2 - 1
H3K4me3 - 1
H3K9me3 - 1
H3K27me3 - 1
ENCODE
Histone Marks
Enhancer
3kbp

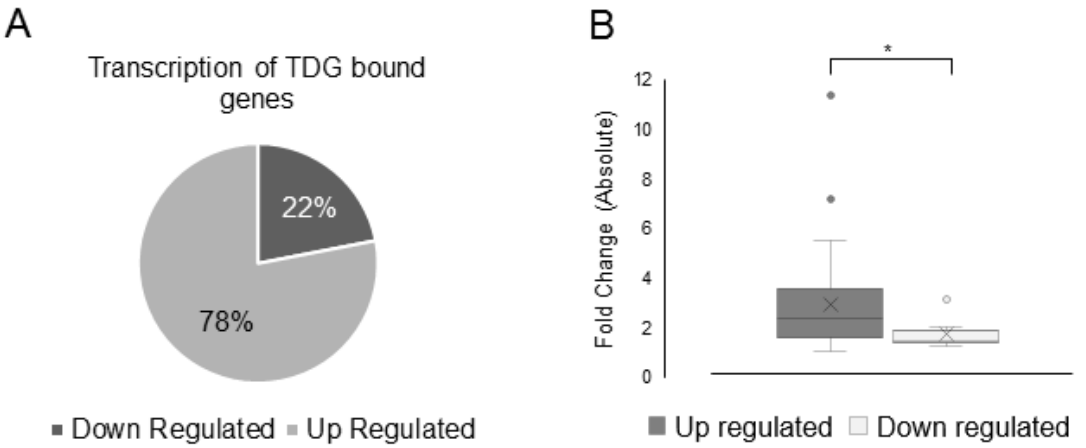

SIAH2
Antibody E2 Rep.
TDG - 1
TDG - 2
TDG + 1
TDG + 2
ERα - 1
ERα + 1
RNA-Pol II - 1
RNA-Pol II + 1
Gata3 - 1
HA_E2F1 - 1
TCFL2 - 1
ZNF217 - 1
P300 - 1
H3K27Ac - 1
H3K4me2 - 1
H3K4me3 - 1
H3K9me3 - 1
H3K27me3 - 1
ENCODE
Histone Marks
Enhancer
7.5kbp

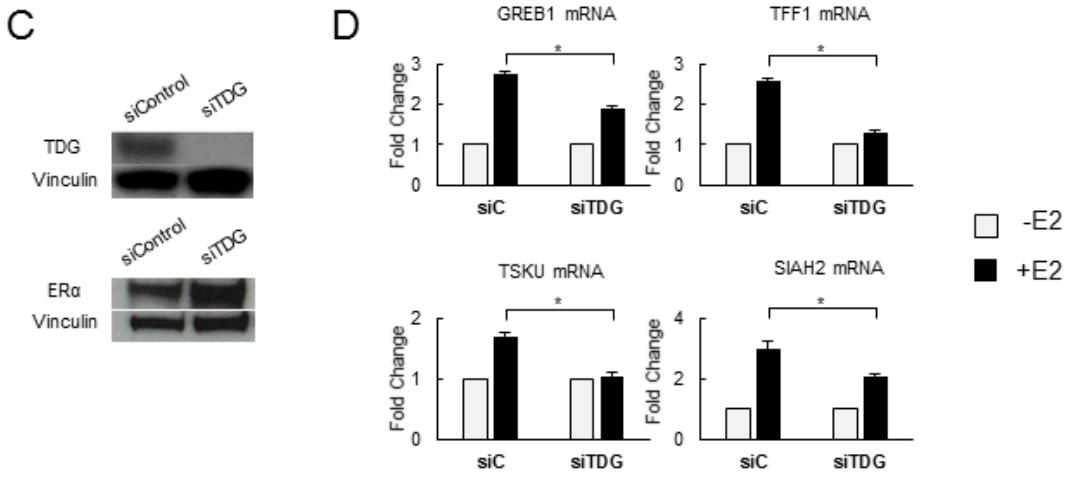
2.2.2 TDG depletion disrupts E2 mediated transcription of ERα target genes

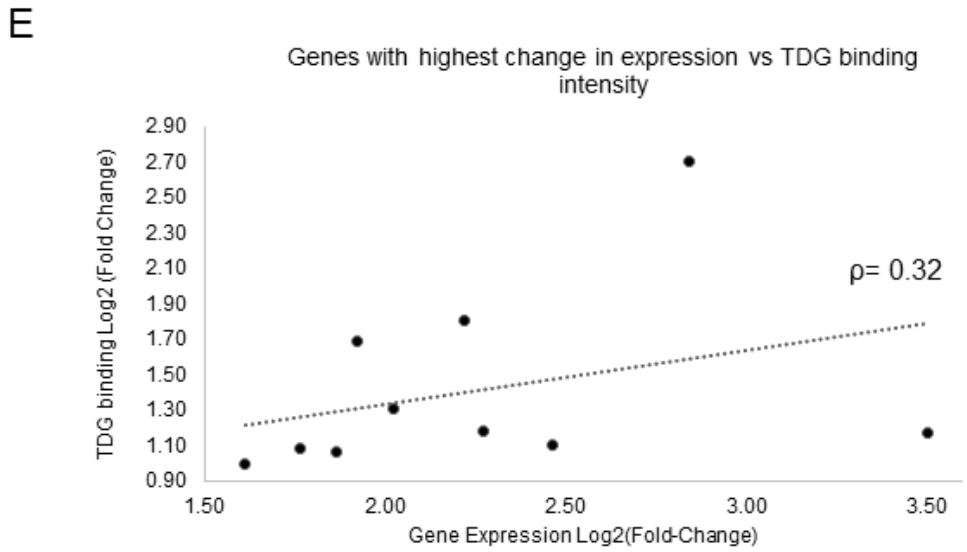
Due to its role as a transcriptional co-activator, I sought to determine whether genes to which TDG binds, in response to E2, are up- or down-regulated. To address this, I identified genes adjacent to TDG binding sites and then obtained their transcriptional response to E2 from publicly available data (Putnik et al., 2012). I find that genes which are differentially expressed in response to E2, and to which TDG localizes, are most often up regulated and the magnitude of change is significantly higher in genes that are upregulated when compared to those that are down regulated (Figure 2-4A and B). To determine whether TDG is critical to the E2-dependant changes in expression, I first treated MCF7 cells with siRNA targeting TDG and immunoblotted for TDG and ERα to ensure ERα levels remained stable during TDG depletion. I then treated MCF7 cells previously treated with scrambled siRNA or siRNA targeting TDG with 100 nM E2 for 1hr and measured mRNA levels of a subset of target genes using qPCR. Remarkably, I find that TDG depletion does not affect levels of ER, yet significantly reduces E2-mediated increase in the transcript levels of all ERα dependent target genes tested. (Figure 2-4C and D). A look at the top 10 genes that bind both TDG and are expressed upon E2 treatment reveals a slight correlation between magnitude of TDG binding and gene expression (Figure 2-4E).

Figure 2-4. TDG is required for E2 dependent gene expression.

TDG peaks were mapped to genes using GREAT software and cross-referenced with publicly available MCF7 E2-dependant expression data. (A) Most genes associated with TDG binding undergo up-regulation in response to E2 and (B) the fold-change experienced by up-regulated genes in response to E2 is greater in magnitude than the fold-change experienced by down-regulated genes (box-and-whisker plot). (C) Western blot of TDG and ERα levels after treatment with scrambled siRNA (siControl) or siRNA targeting TDG (siTDG) (left panels). (D) To determine whether TDG was important for transcriptional upregulation of these genes, siControl or siRNA targeting TDG were treated with 100 nM E2 for 1h. Analysis of mRNA levels using qPCR revealed that loss of TDG decreases, and in some cases completely abrogate, E2-mediated transcription ($n=3$, $p\text{-value} < 0.05$). (E) Comparison of E2-mediated TDG binding at genes which also experience the greatest increase in E2-mediated transcription (Spearman coefficient shown).


A
Transcription of TDG bound genes
22%
78%
Down Regulated
Up Regulated
B
Fold Change (Absolute)
12
10
8
6
4
2
0
*
Up regulated
Down regulated


C
siControl
siTDG
TDG
Vinculin
siControl
siTDG
ERα
Vinculin
D
GREB1 mRNA
TFF1 mRNA
TSKU mRNA
SIAH2 mRNA
Fold Change
siC
siTDG
-E2
+E2

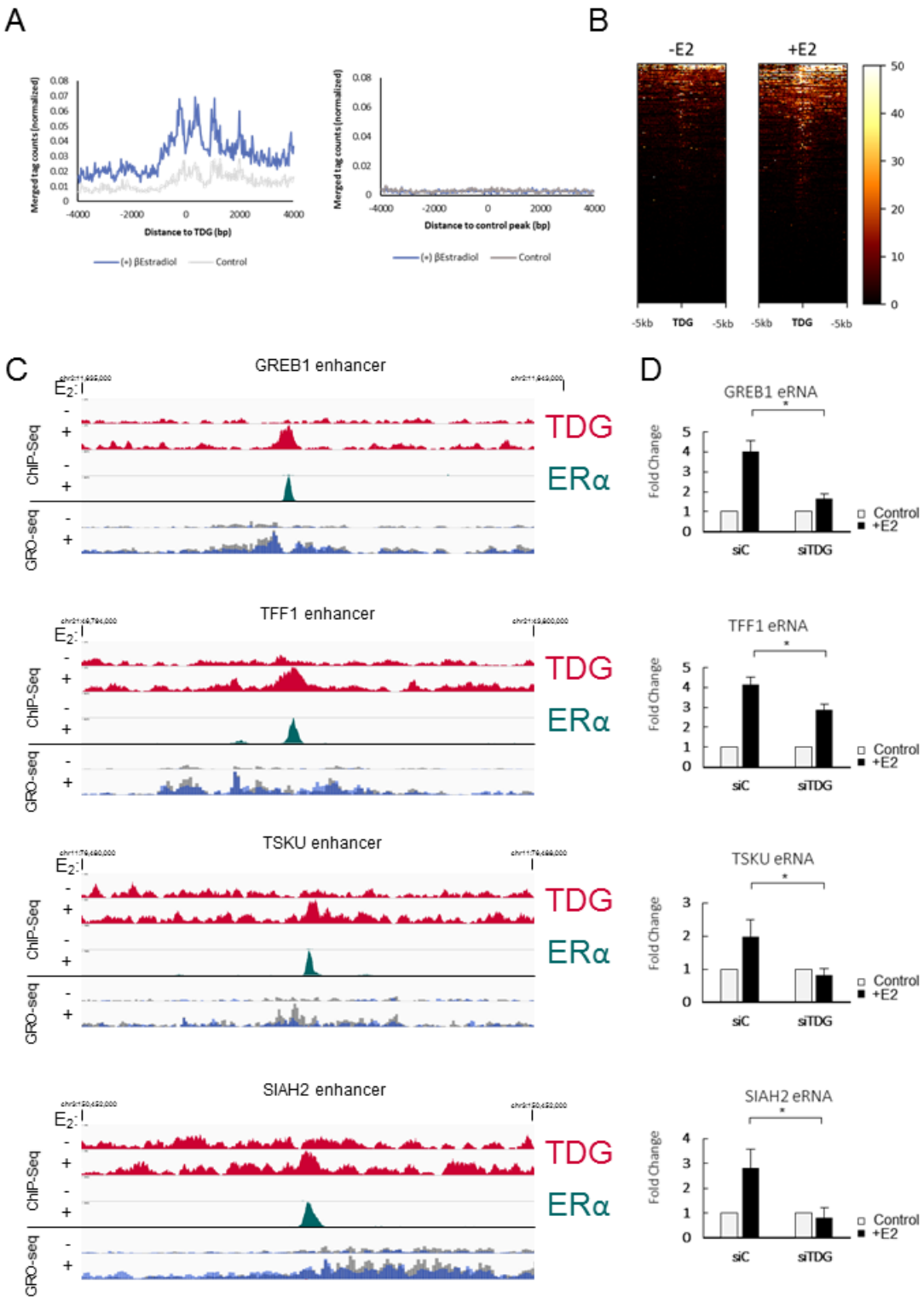

E
Genes with highest change in expression vs TDG binding intensity
TDG binding Log2 (Fold Change)
Gene Expression Log2(Fold-Change)
ρ= 0.32
2.90
2.70
2.50
2.30
2.10
1.90
1.70
1.50
1.30
1.10
0.90
1.50
2.00
2.50
3.00
3.50

2.2.3 TDG is required for eRNA production

Recent studies using Global Run-On Sequencing (GRO-Seq) characterized nascent transcription in response to E2 treatment in MCF7 cells and showed that many of the ERα bound enhancers bind RNA pol II and transcribe enhancer RNAs (eRNA)(Li et al., 2013). Importantly, eRNA transcription and/or eRNA transcripts *per se* are required for activation of adjacent target genes(Li et al., 2013). To determine whether TDG plays a role in eRNA transcription, I first looked too see whether sites of TDG binding coincide with sites of E2-mediated eRNA transcription in MCF7 cells by overlaying sites of E2 dependent TDG localization with publicly available GRO-Seq data. I find that, on average, sites of E2-dependent TDG localization also undergo a concomitant increase in transcription in response to E2 (Figure 2-5A and B). Furthermore, sites of TDG binding at the enhancers of target genes I examined previously, overlap precisely with locations that undergo transcription at those targets (Figure 2-5C). Transcription of non-coding RNA from ER-targeted enhancers is readily induced by 100 nM E2 treatment for 1hr. Remarkably, depleting TDG protein using siRNA prior to treatment abrogates the ability of E2 to induce eRNA from TDG-targeted enhancers (Figure 2-5D). These findings reveal for the first time a potential mechanism by which TDG regulates ER-signaling.

Figure 2-5. TDG depletion impacts eRNA production.

(A) Publicly available GRO-Seq data looking at levels of transcription at sites of E2-dependent TDG binding reveals that sites of TDG binding experience increase in transcription in response to E2 (a set of random peaks reflecting precisely the size distribution of actual TDG peaks was used as control). (B) Heatmap of nascent transcription using publicly available GRO-Seq data at sites of TDG -/+ E2. (C) E2 effects on localization of TDG and ER, as well as transcription response, at specific targets. (D) To determine whether TDG is required for eRNA production, MCF7 cells were depleted of TDG using siRNA and transcript levels were measured in response to 100nM of E2 (1h) (qPCR, p-value < 0.05).

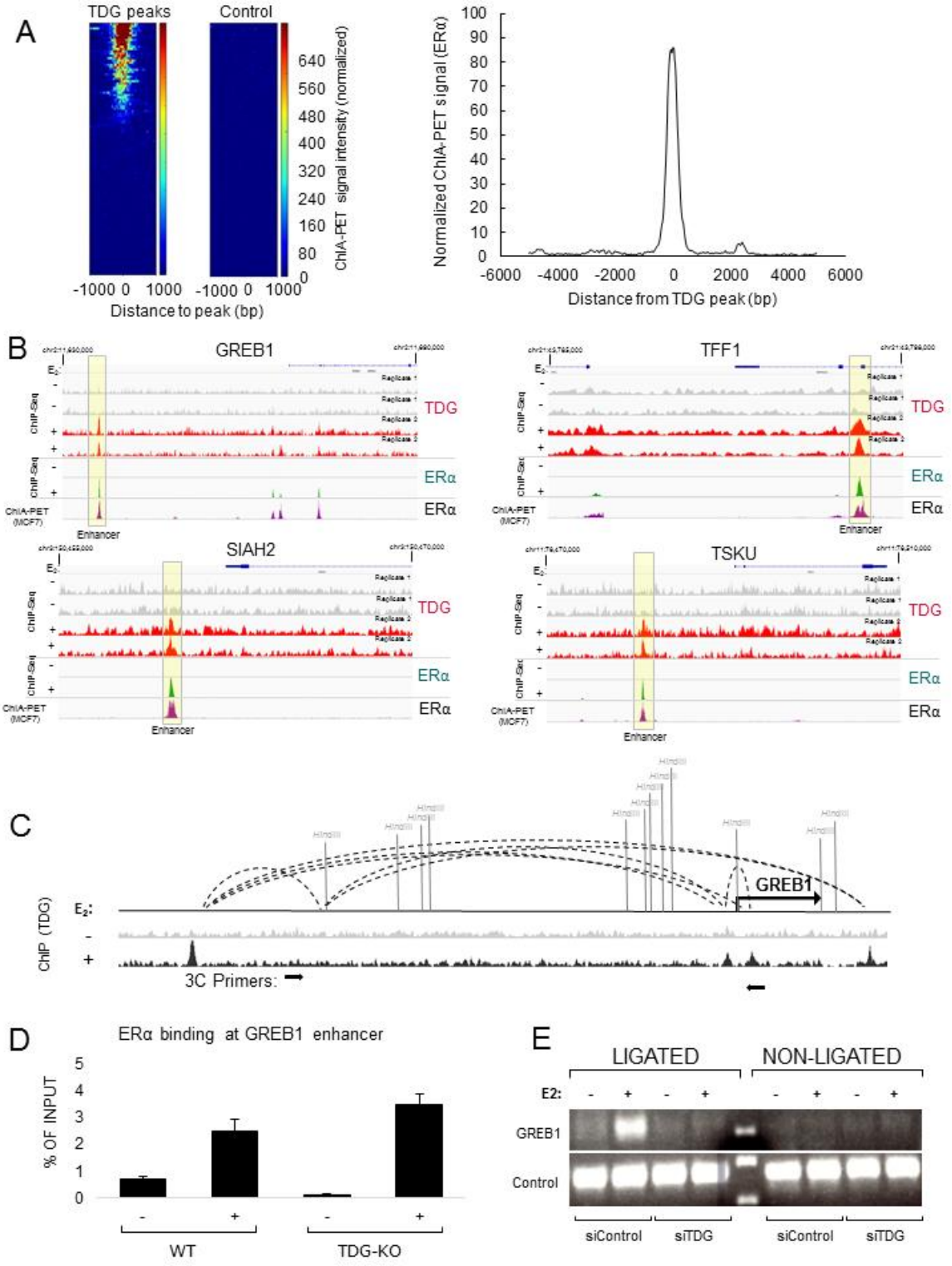

A
Merged tag counts (normalized)
Distance to TDG (bp)
(+) βEstradiol
Control
Distance to control peak (bp)
B
-E2
+E2
-5kb
TDG
C
GREB1 enhancer
TFF1 enhancer
TSKU enhancer
SIAH2 enhancer
ChIP-Seq
GRO-seq
TDG
ERα
D
GREB1 eRNA
TFF1 eRNA
TSKU eRNA
SIAH2 eRNA
Fold Change
Control
+E2
siC
siTDG

2.2.4 TDG is required for 3D conformational changes

Recent work in both ERα and androgen-receptor mediated signaling has revealed that eRNA transcription and/or eRNA transcripts facilitate 3-dimensional re-organization of the genome bringing the enhancer regulatory region into proximity with the promoter and activating optimal target gene transcription (Hsieh et al., 2014; Li et al., 2013). Chromatin Interaction Analysis by Paired-End Tag sequencing (ChIA-PET) is a technique used to capture and quantitate long-range chromatin interactions that occur in the presence of a protein of interest. By comparing ER-dependent TDG binding to data obtained from ChIA-PET looping that occurs at sites of ERα binding in MCF7 cells, I find that a large component of E2-mediated TDG binding occurs precisely at genomic sites that are involved in the interactions between promoter and enhancer (Figure 2-6 A and B). Previous groups have reported that eRNA production at GREB1 is a critical mediator of long-range looping and targeted eRNA degradation is itself enough to attenuate looping and enhancer-complex formation at this gene (Figure 2-6C) (Li et al., 2013). Based on our findings that TDG depletion inhibits E2 driven production of eRNA at GREB1, I predicted that TDG depletion may negatively impact the long-range loop formation. To explore this possibility, I induced formation of looping at GREB1 by treating cells with 100 nM E2 for 1hr after siRNA-mediated depletion of TDG. Similar to previous reports I find that E2 is able to induce the formation of the enhancer-promoter loop at GREB1. Remarkably, ERα continues to be recruited to enhancer in the absence of TDG (Figure 2-6D), but 3D reorganization is abrogated when TDG is depleted, highlighting TDG's impact not only on eRNA production but also on 3-dimensional chromosomal rearrangement (Figure 2-6E).

Figure 2-6. TDG peaks overlap with sites involved in promoter-enhancer looping.

TDG binding was compared to public datasets containing E2-dependent ERα localization and ChIA-PET performed using an antibody against ERα. (A) Heatmap showing ChIA-PET signal at sites of TDG binding (left) and aggregate plot showing global average (right). (B) Overlap of TDG, ER, and ChIA-PET signal at specific sites reveals TDG, ERα and looping occur at precisely the same locations at these sites. (C) Schematic of GREB1 showing approximate locations of looping as identified by publicly available data. (D) ChIP using ERα in the presence and absence of TDG as well as – or + E2, showing that ERα binding is unaltered during depletion of TDG. (E) Loss of TDG prevents enhancer-promoter looping at the GREB1 locus. MCF7 cells were treated with siControl or siTDG, and then treated with 100 nM E2 for 1hr. 3C, semi-quantitative method of measuring the looping between the GREB1 enhancer and promoter, revealed that E2 driven looping of the enhancer and promoter is disrupted upon TDG knockdown.

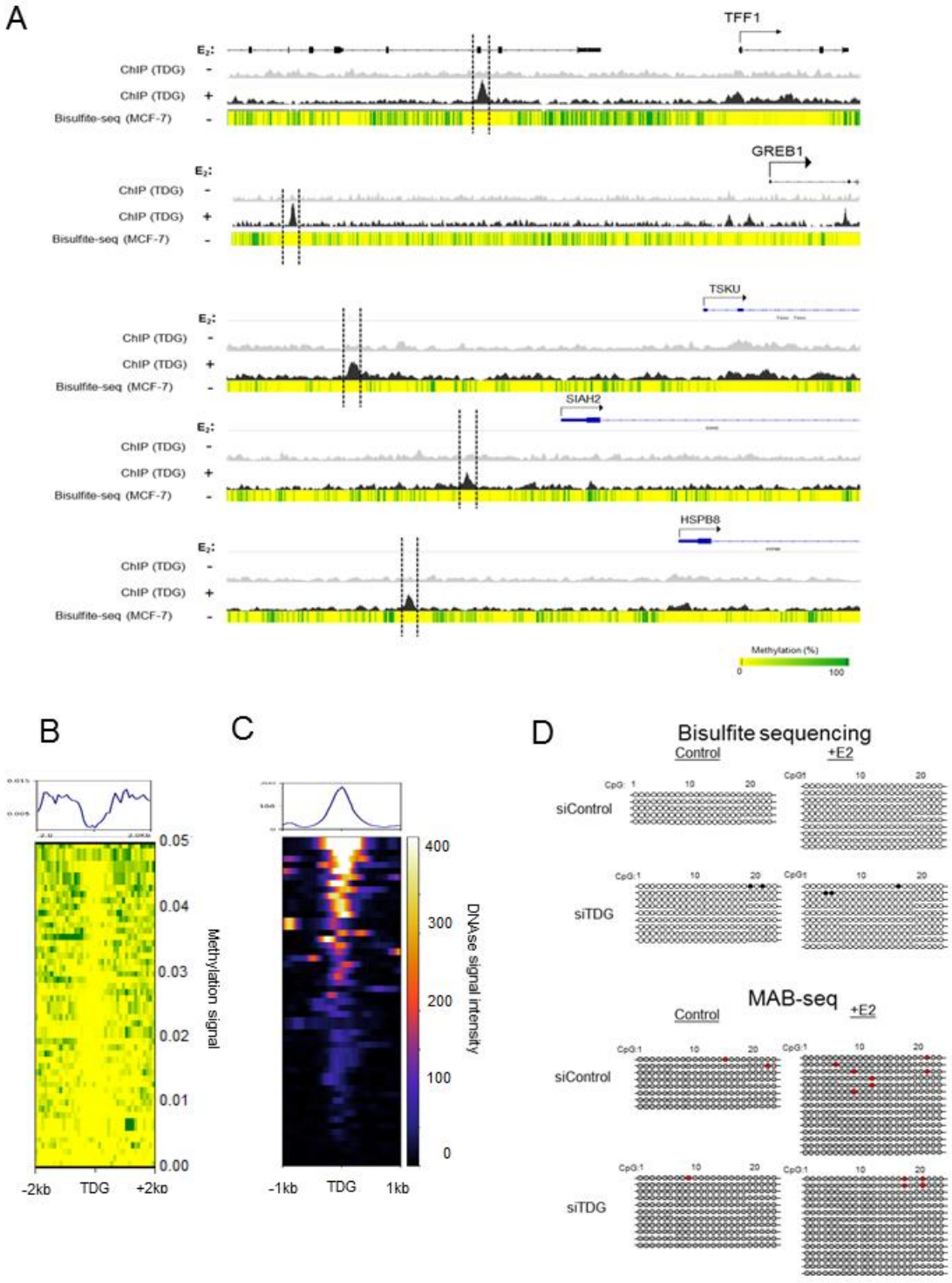

A
TDG peaks
Control
ChIA-PET signal intensity (normalized)
Distance to peak (bp)
Normalized ChIA-PET signal (ERα)
Distance from TDG peak (bp)
B
GREB1
TFF1
SIAH2
TSKU
TDG
ERα
ChIP-Seq
ChIA-PET (MCF7)
Enhancer
C
GREB1
ChIP (TDG)
3C Primers:
D
ERα binding at GREB1 enhancer
% OF INPUT
WT
TDG-KO
E
LIGATED
NON-LIGATED
E2:
GREB1
Control
siControl
siTDG

2.2.5 Methylation status is not impacted by TDG knockdown

Recent studies have shown that the 5mC derivatives 5caC and 5fC, generated during active demethylation, accumulate at 'open' enhancers in TDG knockout MEFs (Raiber et al., 2012; Song et al., 2013) suggesting that active demethylation may be important for eRNA production. Comparing sites of E2-dependent TDG binding with publicly available bisulphite sequencing and DNase data revealed that while TDG binding coincides with 'open' genomic regions, the CpG's are not methylated and the regions are in an 'open' state (Figure 2-7A-C) (Menafra et al., 2014). Bisulfite sequencing is unable to distinguish between unmethylated cytosine and 5fC/5caC. To obtain a clearer picture of what impact E2 signaling and TDG may have on 5mC derivatives at enhancers, I performed Methylase-assisted bisulfite sequencing (MAB-Seq). MAB-Seq consists of pre-treating genomic DNA with the bacterial methyltransferase enzyme *M.SssI*, which methylates unmodified cytosines (C). The *M.SssI* treated DNA is then treated with bisulphite which converts 5fC and 5caC to thymine (T) but does not convert cytosines (which have been converted upstream to 5mC by *M.SssI*). Therefore, sequencing would indicate 5fC and 5caC as T, whereas C/5mC/5hmC would be sequenced as C. Comparing bisulphite sequencing with MAB-Seq results confirmed that TFF1 and GREB1 enhancers are composed almost entirely of unmethylated cytosines and are not altered either in response to E2 treatment or TDG depletion indicating that active demethylation at specific enhancers is not required for E2 signaling (Figure 2-7D).

Figure 2-7. Cytosines in TFF1 and GREB1 enhancers are epigenetically unmodified.

(A) Comparing TDG binding at sites regulated by E2 reveals that TDG binds to hypomethylated locations. (B) Heatmap comparing methylation signal at sites of TDG binding globally, reveals that TDG binds to hypomethylated sites. (C) MCF7 DNase signal intensity, revealing that, globally, TDG binding occurs preferentially at sites which are hypomethylated and 'open'. (D) Bisulfite-sequencing and MAB-Seq at TFF1 after cells were treated with scrambled siRNA (siControl) or siRNA targeting TDG (siTDG) and then with or without E2 treatment. TFF1 enhancer is devoid of any methylation or active-demethylation metabolites and remains so in response to E2 or TDG depletion.


A
TFF1
GREB1
TSKU
SIAH2
HSPB8
E2:
ChIP (TDG) -
ChIP (TDG) +
Bisulfite-seq (MCF-7) -
Methylation (%)
0
100
B
Methylation signal
0.05
0.04
0.03
0.02
0.01
0.00
-2kb
TDG
+2kb
C
DNAse signal intensity
400
300
200
100
0
-1kb
TDG
1kb
D
Bisulfite sequencing
Control
+E2
siControl
siTDG
MAB-seq
Control
+E2
siControl
siTDG

2.2.6 TDG knockdown affects cell proliferation of MCF7 breast cancer cells

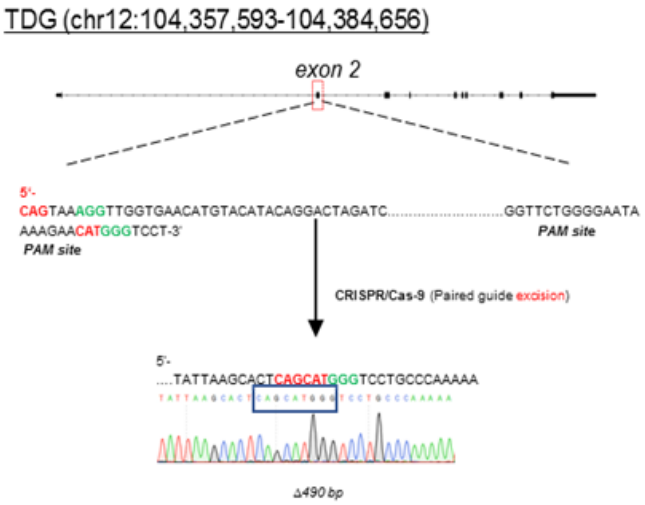
Gene Ontology (GO) enrichment analysis was performed on genes which bind TDG and are upregulated in response to E2. I found a significant enrichment at GO terms directly related to proliferation, including “regulation of epithelial cell proliferation”, as well as multiple terms implicating a role in “differentiation” and “Wnt signaling”. Wnt signaling was a particularly interesting finding as TDG has recently been shown to directly upregulate components of Wnt signaling pathway in colorectal cancer (CRC), and TDG depletion inhibited proliferation of CRC cells both *in vitro* and *in vivo* (Xu et al., 2014). To determine whether TDG plays a role in E2-dependent cell proliferation I deleted TDG from MCF7 cells constitutively using CRISPR technology, or transiently using siRNA (Figure 2-8). In both systems, TDG depletion significantly decreases the E2-mediated increase in proliferation compared to control cells, while also increasing sensitivity to the anti-estrogen tamoxifen, with TDG depleted cells exhibiting a stronger cytostatic response than controls (Figure 2-9A).

Estrogens and the anti-estrogens, such as tamoxifen, can modulate the proliferation capacity of breast cancer cells in part by causing complex rearrangements of both the cytoskeleton and adhesion apparatus (DePasquale et al., 1994; Ma et al., 2014; Millon et al., 1989). Remarkably, I find that TDG depletion in MCF7 cells drastically alters decreases their ability to adhere to the substratum, and to one another (Figure 2-9B and C). This finding is important as anti-estrogens promote an invasive

Figure 2-8. CRISPR-mediated deletion of TDG in MCF7 cells.

(A) To eliminate TDG protein from MCF7 cells I used the CRISPR/Cas-9 and pair guided excision to remove a 490bp region of TDG which contained exon 2 and which also created a frame-shift. (B) Protein levels of TDG in wildtype (WT) cells as well as those edited using CRISPR or siRNA (C: control siRNA or non-targeting CRISPR, TDG: siRNA targeting TDG or CRISPR targeting TDG).

A


TDG (chr12:104,357,593-104,384,656)
exon 2
5'-
CAGTAAAGGTTGGTGAACATGTACATACAGGACTAGATC.....GGTTCTGGGGAATA
AAAGAACATGGGTCCT-3'
PAM site
PAM site
CRISPR/Cas-9 (Paired guide excision)
5'-
....TATTAAGCACTCAGCATGGGTCCTGCCCAAAAA
Δ490 bp

B

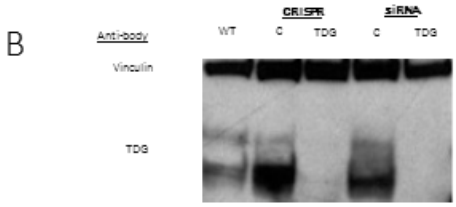
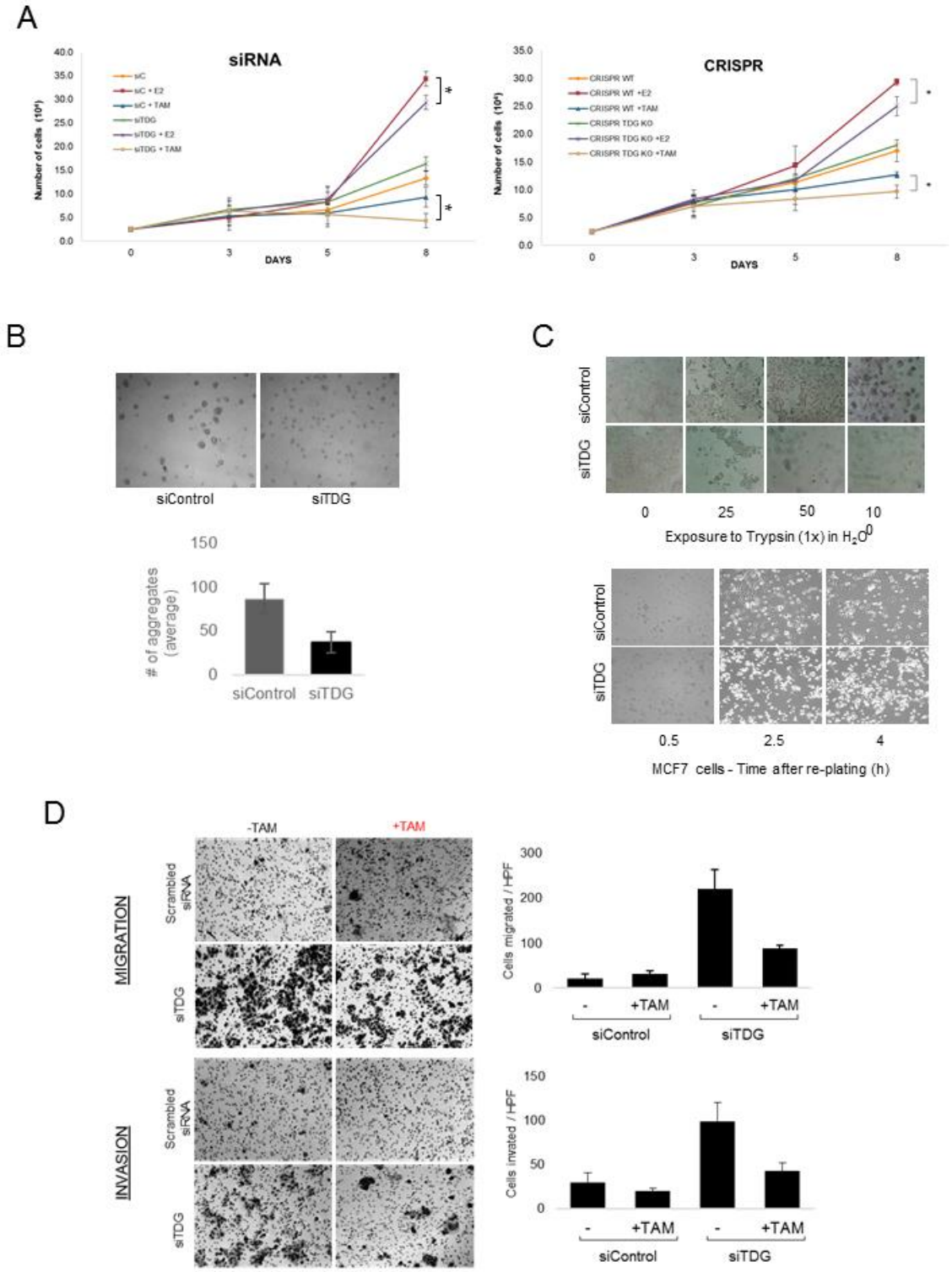

CRISPR
siRNA
Antibody
WT
C
TDG
C
TDG
Vinculin
TDG

Figure 2-9. TDG depletion sensitizes MCF7 cells to Tamoxifen and produces proliferation and attachment deficiencies.

(A) Growth curves examining responsiveness of CRISPR-mediated (upper panel) and siRNA-mediated (lower panel) TDG knockout and knockdown respectively. MCF7 cells were grown for 48-72h in charcoal-stripped phenol-red free media prior to treatment (left panel) (n=3, p-value < 0.05, error bars show -/+ 1 standard deviation). (B) To examine migration and adhesion MCF7 cells were treated with either scrambled siRNA or siRNA targeting TDG and grown in regular media for 2 days prior to performing the cell-to-cell adhesion assay. I found that MCF7s with depleted levels of TDG form significantly less aggregates with one another (n = 4, error bars indicate standard deviation -/+ 1) (C) Treatment of MCF7 cells with varying concentration of Trypsin revealed that those depleted of TDG detached from tissue-plate substratum at lower concentrations (top panel) and upon resuspension TDG depleted cells demonstrated re-attachment deficiencies, remaining loosely attached and spherical while cells treated with siControl became stabilized with visible 'flattening' indicative of cell-substratum contacts(bottom panel). (D) To measure the effect of TDG on the migration and invasion capacity, TDG was depleted in MCF7 cells using siRNA which were treated with or without Tamoxifen for 3d. Cells were harvested using 3mM EDTA and a suspension of 100,000 cells was incubated at 37C for 72 in gelatin or Matrigel to test migration and invasion, respectively. Cells were counted using ImageJ (n=4). MCF7s depleted of TDG show a drastically more aggressive profile with cells migrating and invading gel at a significantly higher rate than siControl cells.


A
siRNA
CRISPR
Number of cells (10⁴)
DAYS
siC
siC + E2
siC + TAM
siTDG
siTDG + E2
siTDG + TAM
CRISPR WT
CRISPR WT +E2
CRISPR WT +TAM
CRISPR TDG KO
CRISPR TDG KO +E2
CRISPR TDG KO +TAM
B
siControl
siTDG
# of aggregates (average)
C
Exposure to Trypsin (1x) in H2O
MCF7 cells - Time after re-plating (h)
D
-TAM
+TAM
MIGRATION
INVASION
Scrambled siRNA
Cells migrated / HPF
Cells invated / HPF

phenotype in breast cancer cells which have adhesion deficiencies (Borley et al., 2008). Specifically, the researchers found that exposure to tamoxifen or fulvestrant promoted invasion in cells maintaining poor cell-cell contacts. To test whether the adhesion defects observed in TDG-depleted MCF7s promote migration or invasion, I depleted TDG in MCF7s using siRNA and treated cells with the anti-estrogen tamoxifen and recorded their ability to transverse gelatin, or Matrigel, respectively. While TDG depletion sensitizes MCF7 cells to the cytostatic effects of tamoxifen, I find that MCF7 cells depleted of TDG become much more aggressive, with significant increases in both migration and invasion capacity (Figure 2-9D).

2.3 Discussion

Utilizing a combination of functional genomic analysis and biological assays, I have identified a role for TDG in E2 dependent signaling in MCF7 breast cancer cells. In response to E2 TDG localizes to distal regulatory sites of ERα target genes. Approximately half of the TDG binding sites identified overlap with sites of E2-mediated ERα binding.

Importantly, in response to E2 TDG localizes to enhancer regions that play an important role in the production of eRNAs and 3-dimensional re-organization important for target gene transcription. By focusing on a subset of TDG target genes whose transcription is upregulated in response to E2 treatment, I found that TDG depletion significantly reduces the ability of E2 to induce transcription of eRNA produced at the enhancers, disrupts looping, and inhibits transcription of the target genes.

eRNA producing enhancers have several common characteristics that include increased binding of transcriptional coactivators, greater chromatin accessibility and increased formation of enhancer promoter looping. Although a direct functional role of eRNAs is still unclear, mounting evidence supports the notion that eRNA production is not merely transcriptional noise as previously suggested but play a functional role by contributing to transcriptional activation of adjacent coding genes. While it remains unclear as to whether eRNA transcription or the eRNA transcript *per se* are responsible for 3-dimensional re-organization that brings the enhancer and promoter into proximity of one another, I have found that TDG depletion disrupts gene transcription broadly, interrupting both eRNA production, 3-dimensional reorganization and activation of target gene transcription. Furthermore, the finding that TDG binding occurs primarily outside of promoters suggests that dynamics at enhancers play an important role in regulating ERα target gene expression.

Previous reports have suggested that stimulation of ERα signaling at some promoters triggers a cyclical methylation/demethylation mechanism involving DNMTs (Métivier et al., 2003, 2008). It was proposed that in addition to functioning as DNA methyltransferases, DNMT3a/b are capable of deaminating 5mC when SAM is limiting. The resulting G:T mispair is then excised by TDG and the base excision repair machinery restores unmethylated cytosine. More recently reports have emerged showing that TDG depletion in embryonic stem cells resulted in the accumulation of active demethylation metabolites 5fC and 5caC at identified enhancer regions (Shen et al., 2013; Wu et al., 2014). To determine whether active demethylation plays a role at enhancers I used MAB-Seq to establish a profile of the active demethylation intermediates 5fC/5caC at the site of TDG binding pre- and post-E_2 treatment and in conjunction with wildtype TDG levels or

with siRNA-mediated depletion of TDG. I found that the TFF1 enhancer appears to be composed entirely of unmodified cytosines regardless of E2 treatment. The observation that TDG depletion in MCF7 breast cancer cells leads to no accumulation of 5fC/5caC supports reports that the glycosylase activity of TDG is dispensable for E2 mediated signaling and instead it is TDGs ability to act as a coactivator that potentiates ERα activity(Chen et al., 2003).

The importance of E2-dependent signaling in breast cancer has been well documented. For example, it has been demonstrated that growth, proliferation and metastatic nature of MCF7 cells transplanted into nude mice are E2 dependent (Kubota et al., 1983). Treatment of MCF7s with either E2, or the anti-estrogen tamoxifen, has been shown to cause changes in proliferation and growth through complex large-scale rearrangements of the cytoskeleton and adhesion apparatus (Borley et al., 2008; DePasquale et al., 1994; Marchisio et al., 1986). Based on our findings that TDG seems to be intimately involved with E2 signaling I investigated whether its role extends to proliferation. I found that deleting TDG from MCF7 cells using either CRISPR technology or siRNA transfection inhibited E2-dependent proliferation. Interestingly, GO analysis revealed that E2 causes TDG binding and upregulation of genes involved in "Wnt signaling", in addition to other proliferation-related categories such as multiple GO terms referencing 'differentiation'. This is consistent with previous studies showing that TDG plays a critical role in the progression of colorectal cancer by upregulating components of Wnt signaling pathway in a CBP/p300 dependent manner. Importantly, researchers observed that TDG depletion significantly inhibited proliferation of CRC cells *in vitro* and *in vivo* (Xu et al., 2014). Taken together, our findings suggest that TDG's role in Wnt signaling may perhaps extend

outside of CRC and play an important role in breast cancer. Further studies will be required to determine to what extent this may be the case.

We have found that TDG is also critical for maintaining proper cell-cell and cell-substratum contacts in MCF7 cells and depletion of TDG leads to broad adhesion defects. This is an important consideration as tamoxifen treatment has been shown to promote an invasive phenotype in ER-positive breast cancers when cell-cell contacts are weak (Borley et al., 2008). Migration and invasion assays have confirmed this, revealing that TDG depletion results in a much more aggressive phenotype with cells demonstrating drastically increased migration and invasion capacity in response to tamoxifen suggesting that TDG possesses tumour suppressive properties despite being a positive regulator of estrogen dependent cell growth. Based on these opposing roles I would predict that cells containing a TDG mutation would not have a selective growth advantage and would be removed before causing genetic and/or epigenetic changes resulting in cancer. This may explain why homozygous mutations for TDG have not been identified in breast cancer based on TCGA data set analysis. Taken together, our findings reveal TDG is important in E2 signaling by regulating eRNA production at ER-targeted enhancers. Furthermore, our functional analysis revealed that TDG plays a critical role in proliferation in response to estrogens and anti-estrogens. Further investigation into the potential for TDG as a therapeutic target is strongly warranted.

2.4 Materials and methods

Cells culture, treatment and transfections

MCF7 cells were obtained from ATCC and grown in low-glucose DMEM and 10% Fetal Bovine Serum (FBS). Prior to treatment, cells were washed once with Phosphate Buffered Saline (PBS) and grown in phenol red-free media containing charcoal stripped FBS (10%) for 72h. Cells were then washed and treated with 100 nM E2 for specific time periods.

For siRNA mediated knockdowns, cells were incubated with Lipofectamine 2000 (LifeTechnologies) and siRNA targeting TDG (Dharmacon, M-040666-01) or scrambled siRNA (Dharmacon, D-001210-03) for 24h, media was replaced with fresh media for 48h at which point experiments were performed.

MCF7 CRISPR TDG-/-

CRISPR TDG-/- and CRISPR TDG+/+ MCF7 cells were generated as previously described using wildtype Cas-9 and two cut sites:

Cut-site 1 (bottom strand): CACCGGTTATTAAGCACTCAGTAA, Cut-site 1 (top strand): AACTTACTGAGTGCTTAATAACC; Cut-site 2 (top strand): (CACCGTCTGGGGAATAAAAGAACAT), Cut-side 2 (bottom strand): AAACATGTTCTTTTATTCCCCAGAC.

Primers used for detection:

Forward(GGCTGACTTGACAGGACTGA),

Reverse(CTGTGCTGAGCTGTAACGTG)(Hsu et al., 2014).

Protein extraction

Whole cell protein extracts were obtained by harvesting cells in RIPA lysis buffer (50mM Tris-pH 8.0, 150mM NaCl, 1% NP-40, 0.1% SDS, and protease inhibitor cocktail), incubating on ice for 15min, followed by centrifugation for 15 min at 4°C (20,000 RCF). Protein concentrations were normalized and proteins were separated using SDS-PAGE and transferred to PVDF membrane. Blocking buffer containing PBS, 0.1% Tween-20 and 5% skim milk powder was used for primary and secondary incubation as well as washes. Protein of interest was visualized using Luminata Forte Western HRP Substrate (Cat. No. WBLUF0100) and by exposure to autoradiography film (GE Healthcare).

RNA extraction, reverse transcription and qPCR

RNA extraction was performed using TRI-zol (Ambion) in accordance with the manufacturer's directions, with additional ethanol wash steps as needed. 2μg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNA levels were ascertained using pre-designed TaqMan probes (Applied Biosystems) targeting the genes of interest, while enhancer RNA levels were monitored using custom designed primers spanning the regions of interest and SYBR Green, per manufacturer's instructions. Experiments were performed with technical triplicates and biological duplicates and run in a 96-well format using the StepOne Real-time PCR System (Applied Biosystems) using GAPDH as a normalization control, unless otherwise noted. Sequences of probes are listed in additional files (see Additional file 12).

ChIP-Seq preparation and analysis

MCF7 cells were serum starved for 3 days, treated with 100 nM E2 for 45min and then ChIP was performed using a polyclonal TDG-targeting antibody that was antigen affinity

chromatography purified (Thermo Fisher Cat. PA5-29140), as previously described, with minor alterations (Thillainadesan et al., 2012). Briefly, cells were cross-linked using 1% formaldehyde in PBS for 10 minutes under shaking at RT. 125 mM glycine in PBS was added for 5min to quench the reaction. Cells were then washed twice with ice-cold PBS and harvested in 1 ml of ice cold PBS buffer. The cells were then pelleted at 250g for 10 minutes, washed twice with ice-cold PBS (protease inhibitors added), and then lysed using 200 µl of lysis buffer (1% SDS, 50 mM Tris-HCl [pH 8.0], 10 mM EDTA, and protease inhibitors) for 15 minutes on ice. The cell lysates were then sonicated, and cell lysate was centrifuged at 15000 rpm for 15 minutes. An aliquot of the supernatant mixture was saved as input DNA, and the remaining lysate was incubated with 5ug of antibody in 50 µl of protein A/G dynabeads as per instructions. Immunoprecipitation was performed overnight at 4°C under rotation. After the immunoprecipitation, the dynabeads were washed twice using wash buffer I (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl), once with wash buffer II (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 500 mM NaCl), wash buffer III (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]) and twice with Tris-EDTA buffer (pH 8.0). The chromatin was eluted using 150 µl of freshly made elution buffer I (1% SDS, 0.1 M NaHCO3) twice at 65°C for 10 minutes. NaCl was added to the eluates and input DNA to a final concentration of 0.3 M and both were incubated at 65°C overnight. Immunoprecipitated DNA was purified using QIAquick PCR Purification Kit (Qiagen) and was analyzed by quantitative PCR following ChIP in technical triplicates and biological duplicates, unless otherwise noted. For high-throughput sequencing immunoprecipitated DNA was sequenced in duplicates at The Centre for Applied

Genomics Next Generation Sequencing facility (Toronto, Ontario). Sequenced reads were mapped to the human genome (hg19) and Partek Genomic Suite was used to call peaks 100 bp bins at an FDR of 0.05. Peaks were further filtered, retaining only those peaks which appeared in both replicates and which showed a greater-than 1.2-fold increase and had a p-value < 0.05 in both replicates.

MAB-Seq

Methylase assisted bisulfite sequencing was performed according to Zhang Y et al. (2014). Briefly, 1 μg of genomic DNA was treated with 4 U of M.SssI in a 20 μl reaction containing 160 mM of SAM. After 2h, the reaction was supplemented with an additional 4 U of M.SssI and 160 mM SAM for an additional 4 hours. This was repeated three times. DNA was purified by conventional phenol:chloroform:isoamyl-alcohol extraction followed by ethanol precipitation after each round of treatment. DNA was then subject to bisulfite conversion, sub-cloned using the TA cloning kit followed by sequencing.

Bisulfite sequencing

DNA was extracted from MCF7 cells using Sigma's Genomic DNA extraction kit and 1 μg was used for bisulfite conversion using the EpiTect Bisulfite Kit (QIAGEN), according to manufacturer's instructions.

Bioinformatics

All datasets used in this study were either based on the hg19 genome or were converted to the hg19 genome using the tool liftOver (http://genome.ucsc.edu/cgi-bin/hgLiftOver).

Mapping peaks to annotated genome was done using Cis-regulatory Element Annotation System (CEAS-Package-1.0.2, http://liulab.dfci.harvard.edu/CEAS/). To mark distance from known transcription sites, Region-gene association graphs were generated using the Genomic Regions Enrichment of Annotations Tool (version 3.0.0) and the following parameters: Association rule: Basal+extension: 5000 bp upstream, 1000 bp downstream, 1x106 bp max extension and curated regulatory domains included.

To determine the relative measure of similarity between E2-dependant TDG localization and that of other transcription factors I downloaded all 690 datasets from the Transcription Factor ChIP-Seq Uniform Peaks from ENCODE/Analysis at UCSC (https://genome.ucsc.edu/ENCODE/downloads.html) and determined the Fisher exact test and the pairwise Jaccard statistic using the Bedtools (2.25.0) options “fisher” and “jaccard”, with default parameters, respectively.

All motif analysis was performed using the latest version of Homer software (version 3.12) (http://homer.ucsd.edu/homer/). Peak visualization was performed using IGV (version/site) with group normalization applied where applicable.

Contrasting TDG localization with gene upregulation was done using GREAT software (using default conditions) to generate a list of genes with which TDG associates and cross-correlating this list with expression data.

GRO-Seq data set was overlapped with sites of TDG binding or control sites (sites which contained the same sized peaks but distributed randomly using the Bedtools (2.25.0) “shuffle” option) using Homer software (v. 3.12) following software guidelines and default parameters. To generate heatmaps of looping at sites of TDG binding publicly available

ChIA-PET data was overlapped with sites of TDG binding or control sites (sites which contained the same sized peaks but distributed randomly using Bedtools (2.25.0) shuffle) using deepTools2 (Ramírez et al., 2016).

Gene Ontology analysis was completed using ConsensusPathDB (http://cpdb.molgen.mpg.de/) using default settings. Cut-off was set at q-value > 0.05.

Chromosome Conformation Capture (3C)

MCF7 cells were treated with 100 nM E2 for 45 min and were cross-linked using 1% formaldehyde for 10 min. The cells were then exposed to Trypsin for 5 min at 37°C followed by 5 min incubation with the 3C lysis buffer at 4°C. 3C was then performed as previously described (Hagege et al., 2007).

Cell-to-cell adhesion

The cell-cell adhesion assay was done as previously described (Rodriguez et al., 2008). Briefly, plates were rinsed twice with PBS and cells were dissociated with 3 mM EDTA. Cells were collected and spun at 400 RCF for 5 minutes, then resuspended in DMEM media and passed through a cell strainer to dissociate cell clusters. Approximately 200,000 cells were added in the appropriate media (DMEM) onto a 6cm petri dish. Plates were incubated at 37ºC on a shaking platform for 30 minutes. After this incubation period, 10 different fields of view were taken per dish at 10x objective. Clusters of > 4 cells were then counted and counts from 10 fields of view were added together for each plate.

For cell-substrate adhesion assays MCF7 cells were grown to confluency and then treated with varying concentrations of Trypsin for 2 minutes, at which point images were obtained.

For re-adhesion assays cells were trypsinized and resuspended in full media (DMEM + 10% FBS) and re-plated onto 6 well. Images were obtained at the times documented.

Migration and Invasion

For migration assays, transwell inserts with 8.0 um pores (Corning, Cat. No. 3422) were coated with 3 µg of gelatin and allowed to dry overnight at room temperature in a sterile environment. The following morning, the gelatin-coated filters were reconstituted with 100uL serum-free DMEM for 90 minutes on a shaker. For invasion assays, transwell inserts with 8.0 um pores were coated with 100 µL of 1mg/mL Matrigel (Corning, Cat. No. 356234) and incubated at 37ºC for one hour to allow for solidification of the Matrigel layer. For both assays, MCF7 cells transiently transfected with siC or siTDG RNA for 3 days prior to being treated with or without tamoxifen. 3 days after treatment, cells were harvested using 3mM EDTA. A cell suspension of 100,000 cells in DMEM + 0.1% BSA was added to the upper well of each transwell insert, and 750uL of DMEM + 10% FBS was added to the lower chamber as a chemoattractant. The cells were incubated at 37ºC and allowed to migrate or invade for 72 h. After this incubation period, the transwell membranes were fixed with 1% glutaraldehyde for 20 min, followed by a 15-min stain with full strength hematoxylin, and brief dip in 1% ammonium hydroxide. Non-migrating and non-invading cells were wiped off the upper surface of the membrane with a cotton swab. Images of 3 non-overlapping fields of view per well were acquired using Image-Pro Analysis Software on an inverted microscope at 10X objective. Cells were counted using ImageJ. Means derived from four replicates were used during analysis.

Primers used in study listed in Table 2-1.

File Sources listed in Table 2-2.

Table 2-1. Primers used in study.

ChIP Primers	
TFF1_eChIP_F	GTTTGTGACCCAGGCATCTT
TFF1_eChIP_R	CAGGGTCCTGTCATTGTGTG
GREB1_ChIP_F	GCTAACCATGCTGCAAATGA
GREB1_ChIP_R	ACACAGTCAGGGCAAAGGAC
SIAH2e_F	ATCCAATTGCTGCAGGTCAC
SIAH2e_R	TCCAGGCAAGGTCACTAAGG
TSKUe_F	TCAGAACGCTCGACCTAGTC
TSKUe_R	TCAGGGCAGGACACATGATT
Neg. Control_F	CATGATTCTCGGGATTTTTCTC
Neg. Control_R	GACAGCTCTGCACCTGTCAT
3C Primers	
GREB1_3C_Bait	GGGTGCTTAGCATGGTACCTGGCAC
GREB1_3C_Enhancer	GATCATACAGTCCCGTCTTCCCTTCCTTCA
Enhancer RNA Primers	
TFF1e_F1	GTTTGTGACCCAGGCATCTT
TFF1e_R1	CAGGGTCCTGTCATTGTGTG
TFF1e_F2	AGGGGATGTGTGTGAGAAGG
TFF1e_R2	GCTTCGAGACAGTGGGAGTC
GREN1eRNA_F1	GCTAACCATGCTGCAAATGA
GREB1eRNA-R1	ACACAGTCAGGGCAAAGGAC
GREB1eRNA_F2	TTGATCTGCTCTTGCCTGAA
GREB1eRNA-R2	GTCCTTTGCCCTGACTGTGT
SIAH2e_F	ATCCAATTGCTGCAGGTCAC

SIAH2e_R	TCCAGGCAAGGTCACTAAGG
TSKUe_F	TCAGAACGCTCGACCTAGTC
TSKUe_R	TCAGGGCAGGACACATGATT
Bisulfite sequencing Primers	
TFF1_enhancer_bis_REV	GAATTGGAGGGGAGTAGTATGAG
TFF1_enhancer_bis_FOR	GACACACCAAAAAACATCCC
GREB1_enchancer_bis_FOR	GGTTTTAAGAGGATTATAAAGAGTG
GREB1_enchancer_bis_REV	CCCACACTTCCAAAATAACAC
Taq-man probes	**Catalogue Number**
GAPDH	Hs02758991_g1
GREB1	Hs00536409_m1
TFF1	Hs00170216_m1
HSPB8	Hs00205056_m1
SIAH2	Hs00192581_m1
TSKU	Hs00539298_s1

Table 2-2. External file sources used in study.

Assay	Target	Treatment	ENCODE	GEO
MCF7 gene expression	N/A	-/+E_2	N/A	GSE36683
MCF-7 Global Bisulfite sequencing	N/A	N/A	N/A	GSE54693
Gro-seq	N/A	-/+E_2	N/A	GSE45822
ChIA-PET	POLR2A	N/A	ENCSR000CAA	N/A
ChIA-PET	CTCF	N/A	ENCSR000CAD	N/A
ChIA-PET	ESR1	N/A	ENCSR000BZZ	N/A
ChIA-PET	POLR2A	N/A	ENCSR000CAA	N/A
ChIP-seq	H3K4me2	N/A	ENCSR875KOJ	N/A
ChIP-seq	H3K4me3	N/A	ENCSR000DWJ	N/A
ChIP-seq	H3K9me3	N/A	ENCSR000EWQ	N/A
ChIP-seq	H3K27me3	N/A	ENCSR000EWP	N/A
ChIP-seq	H3K27Ac	N/A	ENCSR000EWR	N/A
ChIP-seq	GATA3	N/A	ENCSR000EWV	N/A
ChIP-seq	TCF7L2	N/A	ENCSR000EWT	N/A
ChIP-seq	ZNF217	N/A	ENCSR000EWU	N/A
ChIP-seq	EP300	N/A	ENCSR000BTR	GSM1010800
ChIP-seq	HA_E2F1	N/A	ENCSR000EWX	GSM935477
ChIA-PET	ESR1	N/A	ENCSR000BZZ	GSM970212
DNAse-Seq	N/A	N/A	ENCSR000EPJ	GSM736588

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Chapter 3. Role of Thymine DNA Glycosylase in Senescence.

3.1 Introduction

Senescence describes a state of persistent cell-cycle arrest that was originally observed in mammalian cells after excessive passaging (Hayflick and Moorhead, 1961). The cause of senescence was originally attributed to excessive telomere depletion that occurs as a consequence of the 'end replication problem'. Once cells have reached their proliferative potential, telomere depletion results in a complex DNA damage response that ultimately leads to the upregulation of several cell-cycle inhibitors and depletion of pro-mitotic signals, resulting in observed cell-cycle arrest (d'Adda di Fagagna et al., 2003). In addition to prolonged passaging, a wide range of stressors such as hydrogen peroxide treatment (Guo et al., 2010), oncogene activation (Benanti and Galloway, 2004), and exposure to U.V. radiation (Mirzayans et al., 2008) induce senescence in a telomere independent manner. Furthermore, senescence has been observed to play critical roles in many fundamental processes including tissue remodeling, wound healing, aging, and most recently, embryonic development (Muñoz-Espín et al., 2013; Storer et al., 2013; Zhang et al., 2014). Senescent cells differ from their normal counterparts in numerous ways including having an enlarged cytoplasm, being able to cleave X-gal under acidic conditions, and expressing a unique protein profile that includes various tumor suppressors (i.e. p53, p21,ARF) including CKDN2A (Salama et al., 2014). Most cells with an activated senescence program, while non-proliferative, remain metabolically active and exhibit a "Senescence Associated Secretory Phenotype" (SASP), secreting cytokines, chemokines, metalloproteases and growth factors (Pérez-Mancera et al., 2014; Storer et al., 2013). This secretion profile results in inflammation and recruitment of immune cells which then act to clear the senescent and/or damaged cells. Senescence of cells followed by immune

system engagement and clearance is believed to underpin the beneficial effects of senescence in tumor suppression, tissue remodeling and embryonic development. In contrast, the accumulation of senesced cells, or the inability to clear them, has been implicated in the progression of disease, increased tumor aggressiveness, accelerated aging, and other pathologies (Lujambio, 2016).

Although cells undergoing senescence share certain common markers, it has become increasingly clear that the molecular mechanisms that regulate and establish senescence can vary depending on cell-type or context (i.e. what stressor led to the senescence). While therapeutic approaches, both pro- and anti-senescence have been considered, a deeper understanding of the processes that underlie senescence is required to determine under what contexts their use is warranted or appropriate.

A chromosomal region highly implicated in early senescence is the INK4 locus which encodes the cell cycle inhibitors CDKN2A, CDKN2B, and p14ARF. p14ARF inhibits mdm2 which is a negative transcriptional regulator of p53. This causes an increase in the concentration of p53 and induces cell-cycle arrest through the p53 pathway (Williams et al., 2014). CDKN2B and CDKN2A both act by inhibiting CDK4/6, which in turn prevents phosphorylation of Rb, allowing it to remain bound to E2F1, thereby preventing activation of genes required for G1/S transition (Williams et al., 2014). Importantly, CDKN2A has been shown to be critical in the maintenance of senescence and preventing cells from re-entering the cell-cycle (Rayess et al., 2012). The importance of this locus continues to be highlighted by numerous studies showing that the absence of these tumor suppressors, often due to epigenetic silencing or deletion, is tightly correlated with the onset and progression of various cancers corresponding with the loss of senescence and, often times,

poor prognosis (Beauséjour et al., 2003; Bihl et al., 2012; Lou-Qian et al., 2013; Shim et al., 2000; Wang et al., 2012) . Furthermore, epigenetic silencing of CDKN2A involving promoter methylation, has been implicated as a mechanism through which cancer cells can overcome the senescence program (Monasor et al., 2013). In certain contexts, demethylation of the CDKN2A promoter has been shown to lead to its re-expression resulting in cellular senescence (Vogt et al., 1998). Understanding how transcription of these products is regulated is critical to developing our understanding of how cancer overcomes inherent barriers to proliferation and will help us to identify potential therapeutic targets.

Thymine-DNA Glycosylase (TDG), a member of the monofunctional glycosylase family, plays an important role in activating the INK4 protein CDKN2B (Thillainadesan et al., 2012). TDG is a base excision repair enzyme capable of catalyzing the removal of certain mispaired nucleotides (Cortázar et al., 2007). Upon cleaving its substrate, TDG leaves an abasic site that is subsequently repaired by the BER program(Sjolund et al., 2013). Interestingly, TDG associates with other coregulators, such as the Creb binding protein and p300 (CBP/p300)(Tini et al., 2002) as well as steroid receptor coactivators (SRCs)(Hu et al., 2010) and can function as a coactivator by interacting with various transcription factors, such as nuclear hormone receptors (Chen et al., 2003; Um et al., 1998).

The generation of TDG homozygous knockout mice revealed that the loss of TDG results in embryonic lethality at day E11.5 (Cortázar et al., 2011; Cortellino et al., 2011). Importantly, aberrant promoter hypermethylation, concomitant with the emergence of repressive histone marks and loss of activating marks at many genes was observed. Furthermore, the targeting of coregulators and other proteins known to interact with TDG,

such as CBP/p300 was also disrupted. Surprisingly, increases in random DNA mutation due to loss of TDG was not observed (Wu and Zhang, 2014) suggesting that the loss of epigenetic reprogramming during development is likely responsible for the lethality. Complementary studies examining the effects of TDG loss in mouse embryonic stem cells (mESCs) showed an accumulation of TDG substrates (5fC and 5caC) known to be generated through the proposed active demethylation pathway, confirming that loss of TDG impacts active demethylation in development (Raiber et al., 2012; Shen et al., 2013; Song et al., 2013).

Recent studies have shown that mouse embryo's initiate a robust senescence program during development that is maintained from E10.5 to E18.5. During this time, distinct patterns of strongly senescing cell can be observed at various structures including the otic vesicle, fusing neural tube and lining of the limbs. Importantly, mice deficient in genes critical to the initiation and/or maintenance of senescence exhibit defects in senescence patterning and developmental abnormalities at these structures (Muñoz-Espín et al., 2013; Storer et al., 2013; Zhang et al., 2014).

In this study, I explored the role that TDG plays in senescence in two distinct systems: fully differentiated lung fibroblasts as well as in embryonic development. I find that exposing the human lung fibroblast cell-line, IMR-90, to oxidative stress in the form of sublethal doses of H_2O_2, initiates senescence as was evident through cell-cycle arrest, CDKN2A production, and positive β-galactosidase staining. siRNA-mediated Depletion of TDG using siRNA diminishes the H_2O_2-mediated cell-cycle arrest and positive β-galactosidase staining. Importantly, I show that TDG depletion prevents CDKN2A upregulation in response to H_2O_2 and cells depleted of TDG have lower levels of CDKN2A than their wild-

type counterparts. Mechanistically, I show that TDG can bind to the CDKN2A promoter and this binding is significantly increased when cells are expose to H_2O_2. Importantly, I find that TDG is required for the recruitment of CBP/p300, a histone acetyltransferase that plays critical roles in transcriptional upregulation.

To explore whether TDG played any role in developmental senescence I generated a TDG-knockout mouse model and stained embryos for β-galactosidase. Remarkably, I find that TDG-KO and TDG-HET mice exhibit perturbed senescence patterning at specific structures during development. RNA-Seq on whole mount embryos revealed that transcription differences between TDG-KO and WT mice are not global and instead occur at a specific set of genes. However, I show that TDG's role in developmental senescence occurs independent of its role in regulating CDKN2A and using gene-enrichment analysis find that TDG deletion causes a downregulation of genes which are important to establishing senescence and specifically SASP. Taken together our findings suggest that TDG is a key regulator of senescence and may impact senescence in other contexts.

3.2 Results

3.2.1 TDG is required for developmental and H_2O_2-induced senescence.

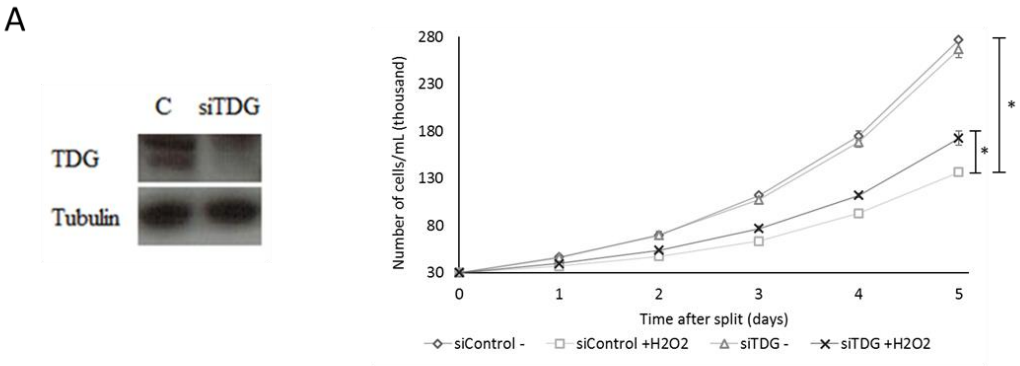
Sublethal doses of H_2O_2 have been previously show to induce senescence in IMR90 human lung fibroblast cells, causing them to cease proliferation and stall in the G1 phase of the cell cycle, with cells exhibit the typical markers of senescence (Chen et al., 1998a). To establish whether TDG plays a functional role in initiation or maintenance of the

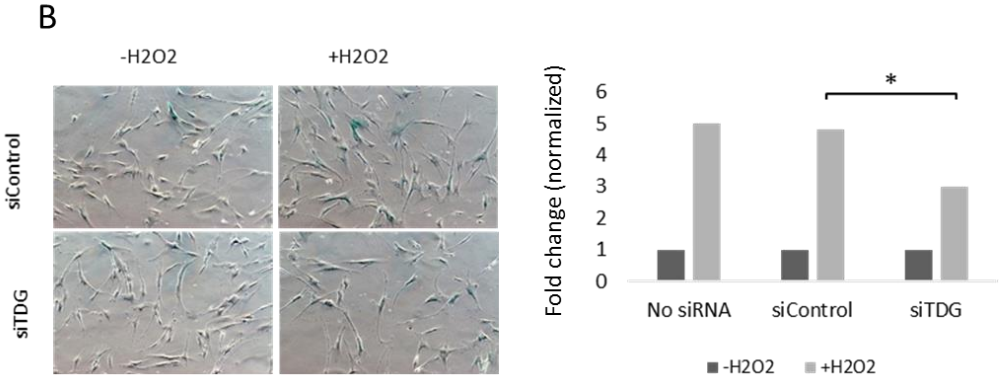
senescence program in fibroblasts I first depleted TDG from IMR-90 cells using siRNA targeting TDG and then treated cells with senescence-inducing concentrations of H_2O_2 and monitored their proliferation. In response to H_2O_2 IMR90s show substantial reduction in proliferation capacity and depletion of TDG increased proliferation compared to controls (Figure 3-1A). In addition to reduced proliferation, a widely used marker for senescence is β-galactosidase activity at an acidic pH. In wild-type cells, β-galactosidase is not active at a pH of 6.0, however cells with an active senescence program exhibit a drastically increased lysosomal mass and increased levels of lysosomal β-galactosidase allowing it to cleave its substrate under acidic conditions (Dimri et al., 1995; Kurz et al., 2000). To test whether the decrease in proliferation response upon TDG depletion is indeed due to a defective senescence program I again exposed IMR90s to sublethal levels of H_2O_2 post TDG depletion and measured the ability to cleave 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), a β-galactosidase substrate (Dimri et al., 1995). While the population of IMR90s already contains a small subpopulation of senescent cells prior to treatment, H_2O_2 causes the number of senescent cells increases approximately 5-fold.

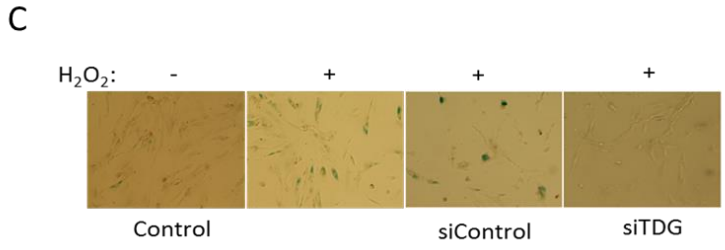
Importantly, consistent with the proliferation assay results, TDG depleted IMR90s show significantly less staining than the controls (Figure 3-1B). I repeated this experiment in another fibroblast cell line (HFL-1 cells) and found a similar dependency on TDG to initiate senescence program, suggesting that the results in IMR90s may extend to other adult tissue as well (Figure 3-1C).

Figure 3-1. TDG is required for proper senescence response in fibroblasts.

(A) IMR90's were treated with either control or H_2O_2, and either siRNA targeting TDG or a scrambled non-targeting siRNA. Left panel – Western Blot. Right panel – Growth curve (Cells were counted every 24 h (n=3)) (B) Left panel - IMR90 cells were treated with siRNA for 3 d then treated with H_2O_2 for 5 d at which point they were stained for the senescence marker β-galactosidase using X-gal**.** Right panel - Manual quantification of staining for IMR-90s. (C) Staining of HFL-1 cells.


A
C
siTDG
TDG
Tubulin
Number of cells/mL (thousand)
280
230
180
130
80
30
0
1
2
3
4
5
Time after split (days)
siControl -
siControl +H2O2
siTDG -
siTDG +H2O2
*
*


B
-H2O2
+H2O2
siControl
siTDG
Fold change (normalized)
6
5
4
3
2
1
0
*
No siRNA
siControl
siTDG
-H2O2
+H2O2


C
H2O2:
-
+
+
+
Control
siControl
siTDG

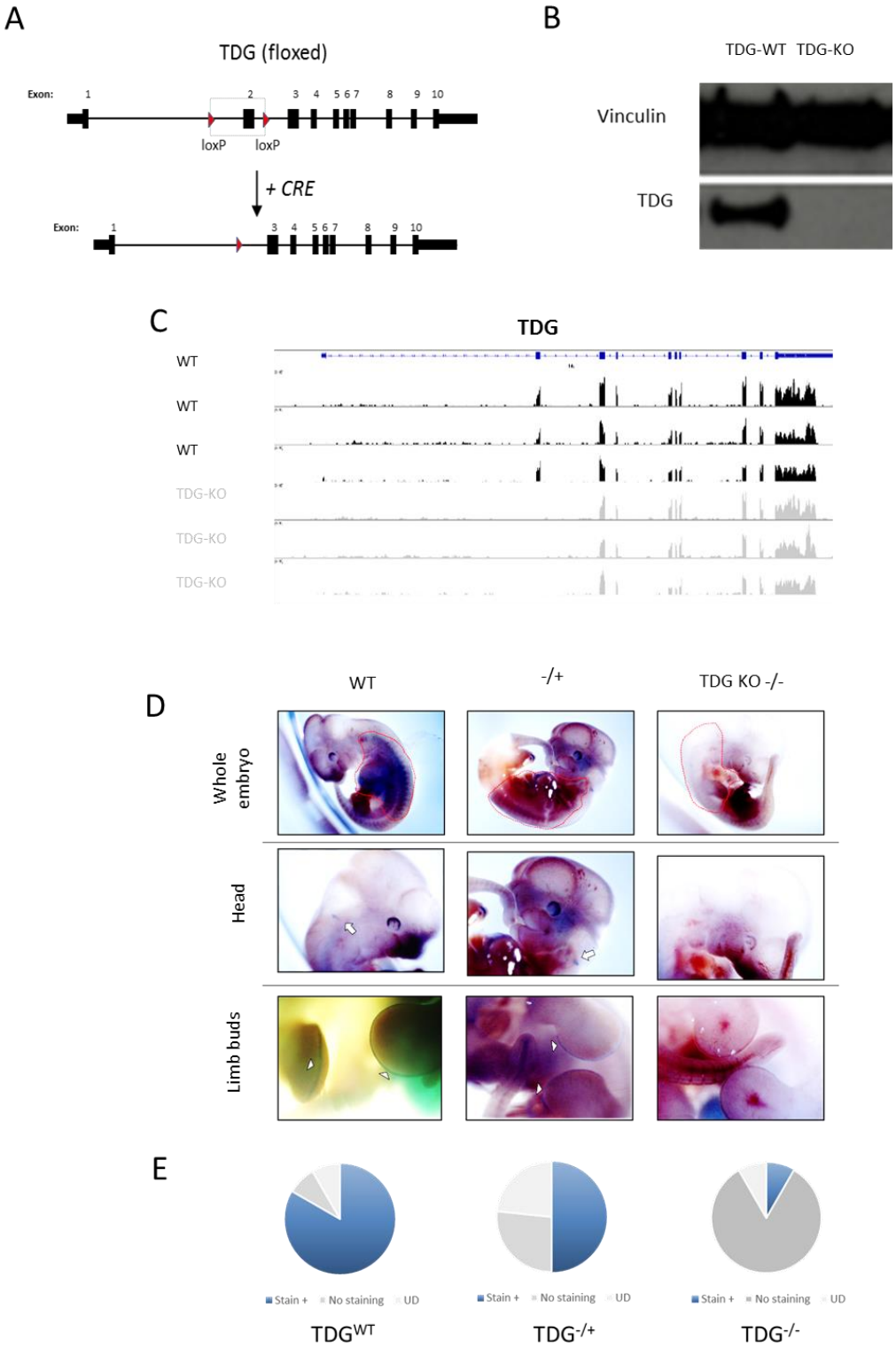
Recent work has revealed that senescence plays an important role in developing mouse embryos(Ewald et al., 2010; Muñoz-Espín et al., 2013; Storer et al., 2013; Zhang et al., 2014). TDG knockout mouse models have previously shown that homozygous deletion of TDG is embryonic lethal, however the role of senescence in these models was not established. To determine whether TDG is important for the establishment of correct developmental senescence patterning, our lab developed mice containing a homozygous and heterozygous deletion of TDG-KO and TDG-HET, respectively (Figure 3-2 A-C). Mice heterozygous for TDG were bred and at E10.5 pups were sacrificed and stained for β-galactosidase activity. Using a double-blind study, I found that wild-type mice display senescence patterning consistent with previous reports showing specific staining of various structures including the ridges of front and hind limbs as well as the otic vesicles. Remarkably, mice depleted of TDG show severe disruptions in a dose dependent manner, with homozygous TDG-KO mice showing more severe disruption, based on staining, than the TDG-HET mice (Figure 3-2D and 2E).

3.2.2 TDG mediates senescence in IMR90s by regulating CDKN2A transcription.

In addition to G1-phase arrest and β-galactosidase staining at pH 6.0, CDKN2A is an established marker that is upregulated in senescent cells (Figure 3-3A). To establish whether TDG regulates transcription of CDKN2A, I treated IMR90 cells with a sub-lethal concentration of H_2O_2, following siRNA mediated TDG depletion, and immunoblotted for CDKN2A. I observed that CDKN2A expression is increased in

Figure 3-2. TDG is required for senescence response during development.

(A) Schematic diagram of the strategy used to create the TDG-knockout mouse using the flox-CRE system (B) Western blot of whole cell lysate of tissue obtained from TDG-WT and TDG-KO embryos. (C) RNA-Seq analysis of the TDG locus in mice. The TDG transcript was disrupted by removing exon 2. (D) β-Galactosidase staining of mouse embryos revealed a defect in senescence in TDG-KO and HET mice in the chest cavity (red perimeter), at the otic vesicle (white arrows) and apical ectodermal ridge. (H) Staining at the otic vesicles, as well as both front and back limb buds on both sides of the body was recorded and shown as a percentage of total (n = 10, N/A = indeterminate due to absence of structure or inconclusive staining).


A
TDG (floxed)
Exon: 1 2 3 4 5 6 7 8 9 10
loxP loxP
+ CRE
Exon: 1 3 4 5 6 7 8 9 10
B
TDG-WT TDG-KO
Vinculin
TDG
C
TDG
WT
WT
WT
TDG-KO
TDG-KO
TDG-KO
D
WT
-/+
TDG KO -/-
Whole embryo
Head
Limb buds
E
Stain + No staining UD
TDG^WT
Stain + No staining UD
TDG^-/+
Stain + No staining UD
TDG^-/-

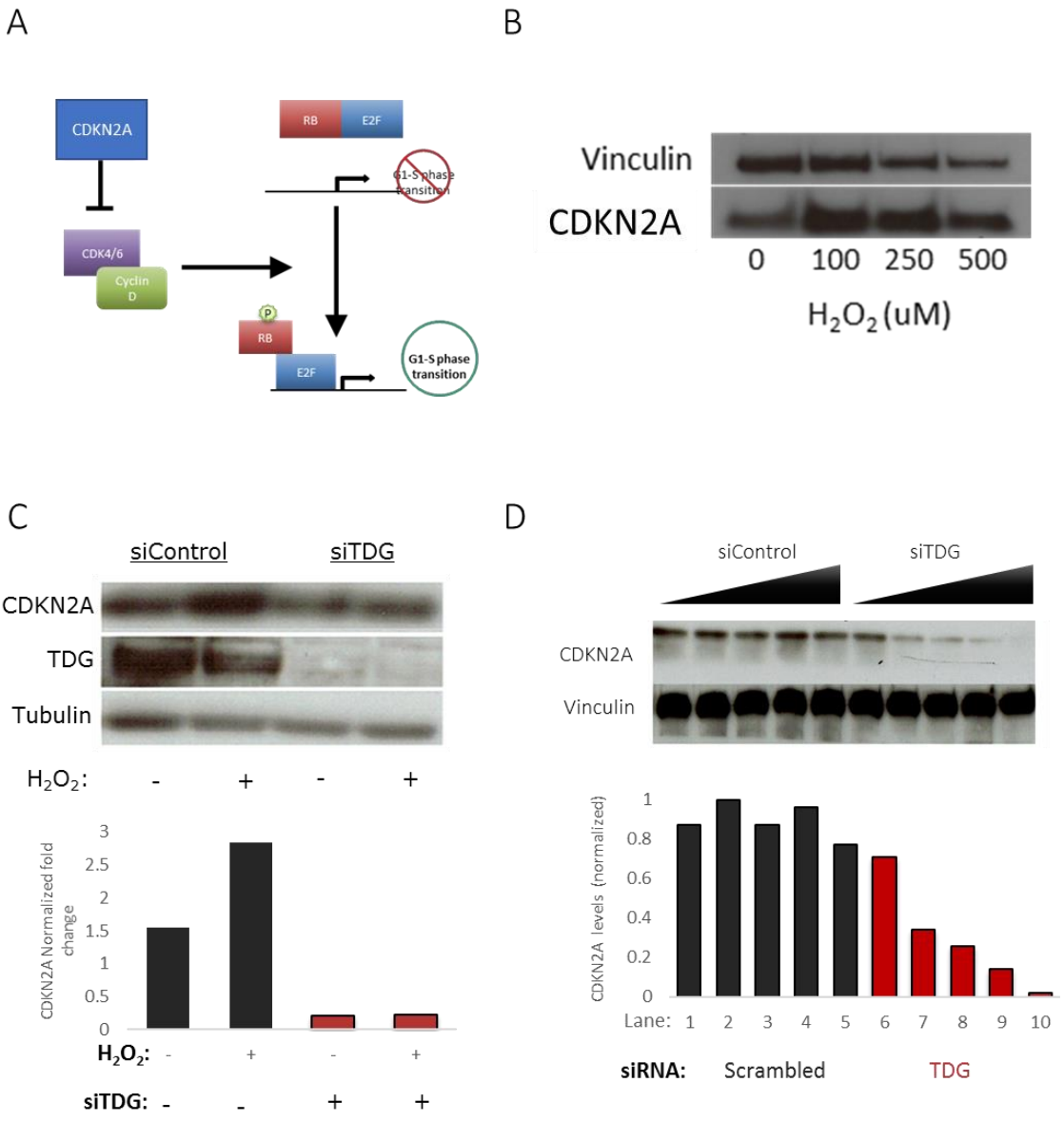
response to H_2O_2, and depletion of TDG prevents the H_2O_2-mediated increase (Figure 3-3B and 3C). IMR90s are typically believed to be a “late passage” cell line and possess “high” basal levels of CDKN2A, consistent with our observations (Benanti and Galloway, 2004). Surprisingly however, TDG depletion not only prevented the H_2O_2-mediated increase in CDKN2A protein levels but cells treated with siTDG exhibited lower levels than our wild-type controls. To explore this further, I titrated the amount of siTDG transfected and observed that CDKN2A levels progressively decrease in response to increased TDG depletion (Figure 3-3D), confirming that CDKN2A expression is dependent on endogenous TDG levels in IMR90s

3.2.3 TDG functions as a Transcriptional Coactivator in IMR90 cells

TDG plays a broad range of roles as a co-activator and mediates the excision of TET catalyzed 5mC metabolites during active demethylation. As a co-activator, TDG has been shown to interact and co-localize with various nuclear receptors and potentiate their transcriptional activity by recruiting additional co-factors (Hassan et al., 2017; Sjolund et al., 2013). Importantly, TDG has been previously shown, in reporter assays, to regulate transcription through the active demethylation of the CDKN2A locus, presumably by directly acting on the locus (Hu et al., 2010). To test whether a similar mechanism governs H_2O_2 dependent transcription of CDKN2A in IMR90 cells, I first performed a ChIP using a TDG-specific antibody followed by qPCR using primers targeting a CpG that overlaps the CDKN2A promoter after treating cells with control or H_2O_2. H_2O_2 treatment causes TDG localization to the CpG island overlapping the

Figure 3-3. TDG regulates CKDN2A expression.

(A) CDKN2A disrupts progression through the cell cycle by inhibiting CDK4/6. This prevents phosphorylation of RB. Rb in turn inhibits E2F from transcriptionally activating genes critical for the cell to progress into S phase. (B) Western blot of CDKN2A. CDKN2A response to varying levels of H_2O_2 was assessed in IMR90s by treating cells with H_2O_2 (C) Top panel – Western blot measuring effects of TDG depletion on H_2O_2 mediated CDKN2A induction. IMR90 cells were treated with siRNA targeting TDG or a control for 3 d followed by treatment with H_2O_2 or control for 5 d. Bottom panel – densitometry of western blot. (D) Top panel - Western blot showing effect of increased concentration of siRNA targeting TDG on endogenous CDKN2a levels in IMR90s. Bottom panel - Quantitation of western blot using densitometry.


A
CDKN2A
RB
E2F
G1-S phase transition
CDK4/6
Cyclin D
P
RB
E2F
G1-S phase transition
B
Vinculin
CDKN2A
0 100 250 500
H2O2 (uM)
C
siControl
siTDG
CDKN2A
TDG
Tubulin
H2O2: - + - +
CDKN2A Normalized fold change
3
2.5
2
1.5
1
0.5
0
H2O2: - + - +
siTDG: - - + +
D
siControl
siTDG
CDKN2A
Vinculin
CDKN2A levels (normalized)
1
0.8
0.6
0.4
0.2
0
Lane: 1 2 3 4 5 6 7 8 9 10
siRNA: Scrambled TDG

CDKN2A promoter (Figure 3-4A-C). To determine whether TDG localization impacts the methylation status of the CDKN2A promoter, I performed bisulfite sequencing on a region of the same CpG island to which TDG localizes. Consistent with the high basal levels of CDKN2A observed in IMR90 cells, bisulfite sequencing revealed that CpGs in the region are hypomethylated and treatment with H_2O_2 or TDG depletion did not significantly change the methylation status (Figure 3-4D). These findings suggested that TDG regulation of CDKN2A levels in response to H_2O_2 is independent of its role in active demethylation.

CBP and p300 are coactivators and lysine acetyltransferases that share a large sequence homology with one another and therefore contain the same set of functional domains and interacting partners. While recent reports have observed differences in the specificity when certain factors (i.e. histones or acetyl-coA) are limited, functionally CBP and p300 are considered to be largely interchangeable, and are often referred to collectively as CBP/p300 (Kasper et al., 2010; Roth et al., 2001).

TDG interacts directly with CBP/p300 and has been shown to colocalize with p300 at many regulatory regions in ES cells (Hassan et al., 2017; Thillainadesan et al., 2012; Tini et al., 2002; Xu et al., 2014). To explore the possibility that TDG dependent induction of CDKN2A involves recruitment of CBP/p300, I treated IMR90 cells with H_2O_2 and performed ChIP-qPCR using a CBP-specific antibody. CBP is indeed recruited to the CDKN2A promoter in response to H_2O_2 and remarkably, TDG depletion using siRNA prevents this recruitment (Figure 3-4E and F). Interestingly, inspection of the CpG island overlapping the CDKN2A promoter revealed a TCF4 binding motif near the site I interrogated for TDG/CBP binding. While it remains to be seen whether TCF4 mediates

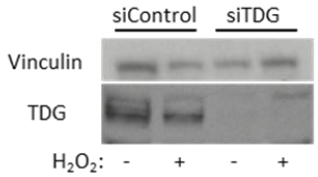
Figure 3-4. TDG's role limited to that of scaffold.

(A) Structure of CDKN2A locus with the primers used indicated. (B) Western Blot showing that while siRNA mediated TDG depletion depletes TDG levels, H_2O_2 does not appear to affect TDG protein levels. (C) To assess whether or not TDG was localized to the CDKN2A promoter I performed a ChIP using TDG-specific antibody and found significant accumulation of TDG in response to H_2O_2 treatment (*, $p<0.05$). (D) Bisulfite sequencing of CDKN2A promoter reveals a relatively unmethylated landscape. (E and F) ChIP and ChIP-qPCR for CBP at CDKN2A. IMR90 cells were treated with siRNA targeting TDG or control for 3 d. Cells were treated with H_2O_2 for 2 h and then allowed to recover for 5 days before ChIP was performed (qPCR, n=3, standard error shown, p-value < 0.05).

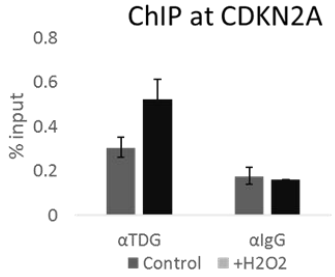
A


CDKN2A
CpG
Chr9:21,974,762-21,974,903

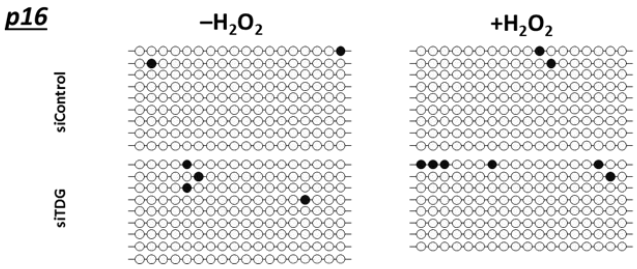
B


siControl
siTDG
Vinculin
TDG
H2O2: - + - +

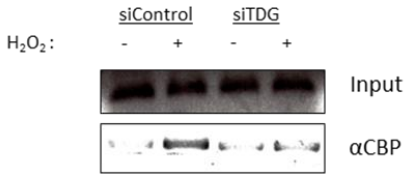
C


ChIP at CDKN2A
% input
0.8
0.6
0.4
0.2
0
αTDG
αIgG
Control
+H2O2

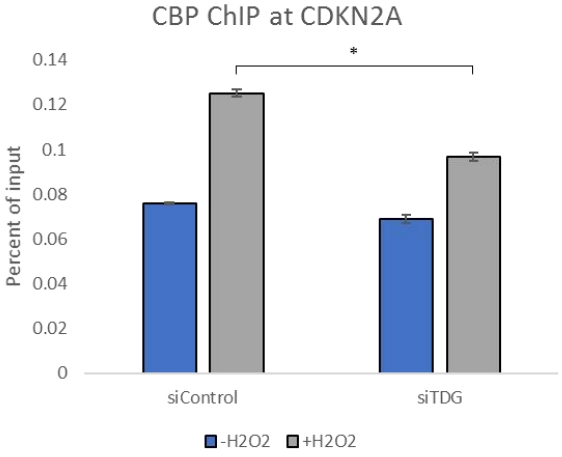
D


p16
–H2O2
+H2O2
siControl
siTDG

E


siControl
siTDG
H2O2: - + - +
Input
αCBP

F


CBP ChIP at CDKN2A
*
Percent of input
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
siControl
siTDG
-H2O2
+H2O2

TDG recruitment in the context of senescence, TDG is able to directly interact with TCF4 and has been shown to be required for TCF4 mediated induction in other systems, such as Wnt signaling in colorectal cancer (Xu et al., 2014). Importantly, TDG interacts with CBP in this context, and target gene induction was concomitant with H3 acetylation of nearby histones (presumably due to CBP recruitment); effects which were lost when TDG was depleted (Xu et al., 2014). TCF4 and CBP have also been implicated in activating CDKN2A transcription, however whether this was through direct binding of the CDKN2A promoter by TCF4 is unclear (Saegusa et al., 2006). Finally, my own work on TDG's role in ER-signaling found that one of the most common binding sites overlapping that of E2-dependent TDG binding in MCF7s, is also TCF4 – suggesting that the relationship between TDG and TCF-4 may span different biological contexts, perhaps even the induction of senescence. Collectively, our findings point to TDG regulating CDKN2A transcription through its actions as a co-activator, possibly through its recruitment by TCF4, in an active-demethylation independent manner.

3.2.4 TDGs role in developmental senescence is independent of CDKN2A induction.

To explore the mechanism governing TDG's role in developmental senescence I performed an RNA-Seq on mouse embryos from TDG-WT and TDG-KO cohorts. Bioinformatic analysis revealed that statistically significant differences in expression are limited to a small set of genes (Table 3-1 and Figure 3-5A). Consistent with the role of TDG as a co-activator, I find that 89% of the genes which are differentially expressed are downregulated in TDG knockdown mice as compared to the WT mice (Figure 3-5B and C). In contrast to

our experiments in adult fibroblasts, I find that CDKN2A is not transcribed in developing embryos and therefore no changes in CDKN2A levels were detected between our cohorts (Figure 3-5D). These findings suggest that TDG's role in initiating or maintaining senescence is broader than I originally anticipated, possibly controlling senescence during development through different/altered pathways than in adult tissue. Senescence-Associated Secretory Phenotype (SASP) plays a critical role in recruitment of immune cells in order to clear senesced cells and is believed to be critical in wound healing, tumor elimination, or tissue remodeling (Lujambio, 2016). More recently the pathway has been shown to be active during development in cells of structures undergoing senescence, suggesting that the clearing of senescent cells is an important aspect of tissue remodeling during development (Storer et al., 2013). To determine if TDG plays a role in SASP during development, I performed gene-list enrichment analysis on the set of genes which are differentially expressed between TDG-WT and TDG-KO. I find that the top hits include, "Cellular response to stress", "Oxidative Stress Induced Senescence" and "Senescence-Associated Secretory Phenotype (SASP)" (Figure 3-5E). Pathway analysis showed enrichment for WNT and HEDGEHOG pathways, both of which are dysregulated during developmental senescence (Muñoz-Espín et al., 2013).

To obtain a better sense of potential mechanisms governing the transcription of our gene list, I used the ENCODE database, which contains ChIP information detailing transcription factor and coactivator binding for various transcription factors under different conditions, to explore whether genes in our list are known to be targeted by any other factors.

Table 3-1. Gene list of differentially expressed genes comparing WT to TDG-KO mice.

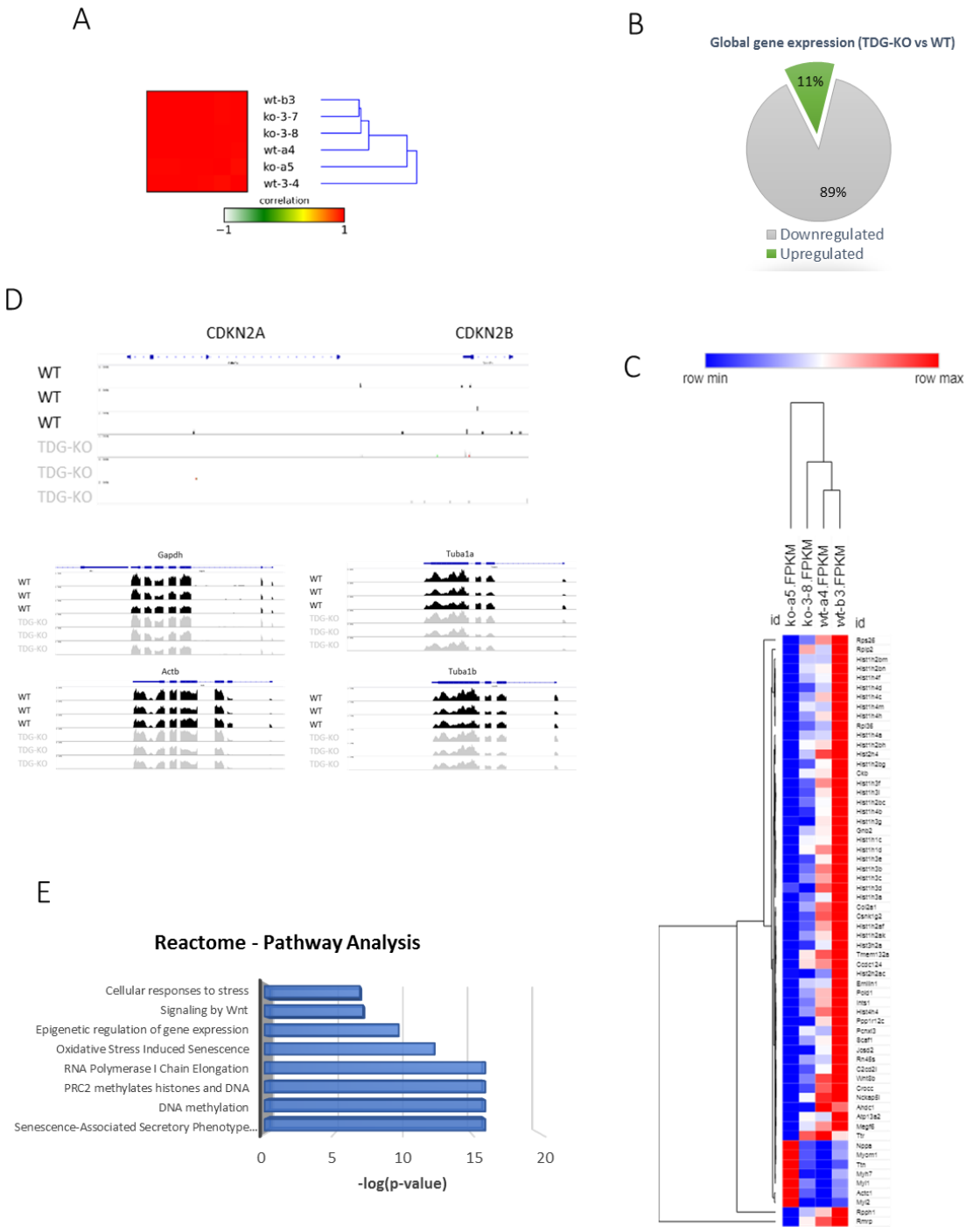
Gene Name	Fold Change (KO/WT)
Hist1h3b	-4.604730112
Rn45s	-4.28755873
Hist1h2af	-3.684236058
Hist1h2ak	-3.67188656
Hist2h2ac	-3.615758371
Hist1h4d	-2.972867629
Hist1h3c	-2.860687465
Hist1h4a	-2.823992808
Hist1h4f	-2.628734762
Hist1h2bc	-2.584279348
Hist1h3e	-2.565768432
Hist1h4h	-2.561694357
Hist1h2bn	-2.547296724
Josd2	-2.389889399
Hist1h4b	-2.358090726
Hist1h4c	-2.355917092
Ttr	-2.351071426
Hist1h3i	-2.305073585
Hist1h3d	-2.252757216
Hist1h2bg	-2.241319199
Hist2h4	-2.235437136
Hist4h4	-2.209367652
Hist1h4m	-2.198653402
Hist1h3g	-2.192280045
Rpph1	-2.178734496
Hist1h3a	-2.163438152
Hist1h3f	-2.136669521
Rpl36	-2.034155925

Wnt8b	-2.021315877
C2cd2l	-1.986464597
Nckap5l	-1.788602022
Ccdc124	-1.776946992
Hist1h2bh	-1.776436736
Hist1h1c	-1.695497477
Hist3h2a	-1.66625845
Crocc	-1.638163022
Emilin1	-1.631945943
Ahdc1	-1.622061063
Tmem132a	-1.591152977
Atp13a2	-1.581161763
Ppp1r12c	-1.573188929
Csnk1g2	-1.545582514
Hist1h2bm	-1.538673476
Scaf1	-1.536761083
Rmrp	-1.518211721
Megf6	-1.511055323
Hist1h1d	-1.510416071
Pold1	-1.505032589
Rplp2	-1.489131856
Pcnxl3	-1.483877776
Ints1	-1.478801309
Rps26	-1.476415794
Ckb	-1.47591883
Col2a1	-1.452279539
Gnb2	-1.443453794
Myh7	1.660958515
Actc1	1.826025366
Ttn	1.8467956

Myom1	1.953907359
Myl1	2.075705638
Nppa	3.236279189
Myl2	3.328548459

Figure 3-5. Transcription profiles of TDG deficient mice compared to WT.

(A) PCA analysis comparing WT and KO mice found that the global transcription profiles were similar between cohorts and, instead, what I found was that TDG deletion caused a significant change in a small subset of genes. (B) Changes in gene-expression between TDG-KO and TDG-WT mice revealed that most genes underwent down regulation in TDG-KO mice as compared to TDG-WT, consistent with previous reports finding that TDG acts primarily as a co-activator, c. (C) Heatmap looking at gene-expression profiles in WT and KO mice. (D) Transcript levels of CDKN2A and various house-keeping genes in both TDG-KO and WT mice. (E) To determine what pathways were affected by TDG knockdown gene enrichment analysis was performed using the Reactome database (Subset of significant results shown).


A
wt-b3
ko-3-7
ko-3-8
wt-a4
ko-a5
wt-3-4
correlation
−1
1
B
Global gene expression (TDG-KO vs WT)
11%
89%
Downregulated
Upregulated
D
CDKN2A
CDKN2B
WT
WT
WT
TDG-KO
TDG-KO
TDG-KO
Gapdh
Tuba1a
Actb
Tuba1b
C
row min
row max
id
ko-a5.FPKM
ko-3-8.FPKM
wt-a4.FPKM
wt-b3.FPKM
id
E
Reactome - Pathway Analysis
Cellular responses to stress
Signaling by Wnt
Epigenetic regulation of gene expression
Oxidative Stress Induced Senescence
RNA Polymerase I Chain Elongation
PRC2 methylates histones and DNA
DNA methylation
Senescence-Associated Secretory Phenotype...
0
5
10
15
20
-log(p-value)

Remarkably, I find that the highest enrichment came from p300 and SP1: ChIP analysis revealed that 32 (48%) of the genes in our list have been shown to recruit p300, while 39 (59%) have shown to recruit SP1 (Table 3-2). This data suggests that the mechanism governing expression of these genes may be similar to the mechanism governing H_2O_2-mediated expression of CDKN2A in IMR90, however further work is required to test this proposition. Collectively these suggest that TDG is required for developmental senescence, and that its role is independent of CDKN2A regulation.

3.3 Discussion

Senescence, originally shown to be a cellular program that limits proliferative potential, has more recently been shown to be a critical component of various fundamental processes, modulating cellular response to oncogenic stress and reactive oxygen species, wound healing, tissue remodeling and, most recently, embryonic development. While a proper senescence program is able to promote positive outcomes, errors in senescence have been shown to be detrimental to health. Building a comprehensive understanding of how senescence is initiated and maintained is a critical step toward developing therapies that can appropriately modulate senescence in a therapeutic context.

In this study I find that TDG, a base-excision protein with an expanding list of roles, is a critical mediator of distinct forms of senescence. In a model of senescence in the IMR90 human lung fibroblast cell, I demonstrated that depletion of TDG leads to an attenuated response to H_2O_2: effects of H_2O_2 on proliferation and β-galactosidase staining are attenuated and CDKN2A induction abrogated. Furthermore, in response to H_2O_2, I show

Table 3-2. ENCODE transcription factor binding at genes whose transcription was significantly altered in WT vs TDG-KO mice.

The list of gene that were identified as being significantly downregulated upon TDG knockout in our RNA-Seq data were compared to the ENCODE (2014) database using Enrichr software. Bolded are instances of SP1 and p300.

Term	Adjusted P-value	Genes
P300_SK-N-SH	0.000187686	HIST1H2BN;CCDC124;HIST2H2AC;HIST1H3A;NPPA;RPL36;RPLP2;EMILIN1;HIST1H3I;CKB;HIST1H3B;HIST1H3E;INTS1;HIST1H2AK;NCKAP5L;TMEM132A;JOSD2;SCAF1;RPS26;HIST1H4A;HIST1H4B;HIST4H4;GNB2;HIST1H4H;HIST1H2BH;HIST1H2BG;HIST1H4C;HIST1H4D;PPP1R12C;AHDC1;HIST1H4F;HIST1H2BC
SP1_H1	0.000187686	HIST1H2BN;HIST1H2BM;CCDC124;HIST2H2AC;RPPH1;HIST1H3A;POLD1;HIST1H3F;RPL36;HIST1H3G;RPLP2;HIST1H3I;HIST1H3B;HIST1H3D;RMRP;HIST1H3E;NCKAP5L;HIST1H2AK;JOSD2;SCAF1;RPS26;HIST1H4A;HIST3H2A;HIST1H4B;COL2A1;HIST4H4;GNB2;CROCC;HIST1H2BH;ATP13A2;HIST1H2BG;HIST1H4C;PCNXL3;HIST1H4D;PPP1R12C;CSNK1G2;C2CD2L;HIST1H2BC;HIST1H4F
IRF3_HELA-S3	0.000419177	HIST1H2BN;HIST1H2BM;HIST1H2AK;HIST2H2AC;HIST1H4A;HIST1H3A;HIST3H2A;HIST1H3F;HIST1H3G;HIST1H2BH;HIST1H2BG;HIST1H4C;HIST1H3B;PCNXL3;HIST1H3C;HIST1H3D;HIST1H2BC;HIST1H1C
MEF2A_K562	0.000984914	HIST1H2BN;HIST1H2AK;CKB;HIST1H4D;C2CD2L;HIST1H3D;HIST1H2BC;HIST2H2AC
P300_H1	0.001381286	INTS1;TMEM132A;CCDC124;JOSD2;SCAF1;HIST2H2AC;HIST1H4A;HIST1H4B;HIST4H4;GNB2;HIST1H4H;RPLP2;HIST1H3I;CKB;HIST1H4C;HIST1H3B;PCNXL3;HIST1H4D;PPP1R12C;CSNK1G2;HIST1H4F;HIST1H3E
RXRA_GM12878	0.001381286	HIST1H2BM;INTS1;CCDC124;JOSD2;SCAF1;HIST2H2AC;HIST1H4B;HIST4H4;GNB2;HIST1H3F;HIST1H4H;HIST1H2BH;HIST1H4C;HIST1H4D
NF-YB_K562	0.001381286	HIST1H2BN;HIST1H2BM;HIST2H2AC;HIST1H3A;HIST1H1D;HIST1H3F;RPLP2;HIST1H3G;CKB;HIST1H3I;HIST1H3B;HIST1H3C;HIST1H3D;HIST1H1C;HIST1H2AK;NCKAP5L;JOSD2;RPS26;HIST1H4A;HIST3H2A;GNB2;HIST1H2BH;HIST1H2BG;PCNXL3;HIST1H2BC
GTF2B_K562	0.001718723	HIST1H2BN;HIST2H2AC;RPPH1;HIST1H3A;HIST1H1D;RPL36;RPLP2;HIST1H3B;HIST1H3D;HIST1H1C;RMRP;INTS1;HIST1H2AK;NCKAP5L;SCAF1;RPS26;HIST1H4A;HIST3H2A;GNB2;HIST1H4H;HIST1H2BH;HIST1H2BG;HIST1H4D;PPP1R12C;C2CD2L;AHDC1;HIST1H2BC
FOS_GM12878	0.001922636	HIST1H2BN;HIST1H2BM;HIST1H2AK;HIST3H2A;HIST1H3A;HIST1H3G;HIST1H3I;HIST1H2BG;HIST1H3B;PCNXL3;HIST1H3C;HIST1H3D;HIST1H2BC
IRF3_GM12878	0.00274444	HIST1H2BN;HIST1H2BM;HIST1H2AK;HIST2H2AC;HIST1H4A;HIST1H3A;HIST3H2A;GNB2;HIST1H3F;HIST1H3G;RPLP2;HIST1H2BH;HIST1H3I;HIST1H2BG;HIST1H4C;HIST1H3B;PCNXL3;HIST1H3C;HIST1H3D;CSNK1G2;HIST1H3E;HIST1H2BC
NRSF_H1	0.00274444	HIST1H2BN;HIST1H2BM;HIST1H3A;HIST4H4;HIST1H2AK;HIST1H4H;HIST1H4C;HIST1H4D;HIST1H4F;RMRP;HIST2H2AC;HIST1H2BC
JUND_HELA-S3	0.00274444	HIST1H2BN;HIST1H2BM;HIST1H4A;RPPH1;HIST1H2AK;HIST1H4H;HIST1H2BG;HIST1H4C;SCAF1
NFE2_GM12878	0.00274444	HIST1H2BN;HIST1H2BM;HIST1H2AK;CCDC124;SCAF1;HIST2H2AC;HIST1H4A;RPPH1;HIST1H4B;HIST4H4;GNB2;HIST1H3I;HIST1H2BG;HIST1H4C;HIST1H3B;PCNXL3;HIST1H3C;HIST1H3D;HIST1H2BC;RMRP
NF-YA_GM12878	0.00274444	HIST1H2BN;HIST1H2BM;HIST1H2AK;HIST2H2AC;HIST1H3A;HIST3H2A;HIST1H1D;HIST1H3F;RPLP2;HIST1H3G;HIST1H2BH;HIST1H3I;HIST1H2BG;HIST1H3B;PCNXL3;HIST1H3C;HIST1H3D;HIST1H1C;HIST1H3E;HIST1H2BC

STAT3_HELA-S3	0.003883862	HIST1H2BN;HIST1H4A;HIST3H2A;GNB2;HIST1H2AK;HIST1H2BG;HIST1H4C;PCNXL3;HIST1H4D;HIST1H3D;HIST2H2AC;HIST1H2BC
TCF12_SK-N-SH	0.003883862	HIST1H2BN;HIST1H2AK;HIST1H4H;EMILIN1;HIST1H4C;HIST1H3B;HIST1H4D;HIST1H3D
SRF_K562	0.004504858	HIST1H2BN;INTS1;HIST1H2AK;CCDC124;JOSD2;SCAF1;RPS26;HIST1H4A;RPPH1;GNB2;POLD1;HIST1H4H;RPLP2;ATP13A2;PPP1R12C;CSNK1G2
CJUN_HEPG2	0.005594011	HIST1H2BN;HIST1H2BM;RPPH1;HIST4H4;HIST1H2AK;CCDC124;HIST1H4H;RPLP2;HIST1H2BG;HIST1H4C;HIST1H3B;SCAF1
CEBPB_GM12878	0.008449704	HIST1H2BN;HIST1H2BM;HIST1H2AK;CCDC124;HIST2H2AC;RPS26;HIST1H4B;HIST3H2A;HIST4H4;GNB2;HIST1H4H;RPLP2;HIST1H3I;HIST1H2BG;HIST1H3B;PCNXL3;HIST1H4D;HIST1H3D;HIST1H3E;HIST1H2BC
NF-YA_K562	0.009152463	HIST1H2BN;HIST1H2AK;HIST2H2AC;HIST1H4A;HIST1H3A;HIST3H2A;HIST1H1D;HIST1H3F;RPLP2;HIST1H2BH;CKB;HIST1H2BG;HIST1H4C;HIST1H3B;PCNXL3;HIST1H3D;HIST1H1C;HIST1H2BC
BRF2_HELA-S3	0.009152463	RPPH1;RMRP

that TDG localizes to a CpG island overlapping with the promoter of CDKN2A and recruits the co-activator CBP, leading to CDKN2A's transcription. Importantly, no change in the methylation status of the CDKN2A promoter was found in TDG depleted IMR90 cells. Recent work in ESCs found that TDG depletion led to the accumulation of the active demethylation metabolites 5caC and 5fC. Bisulfite sequencing is unable to identify these metabolites, which would register the same readout as unmodified cytosines. It is therefore possible that changes in the metabolite landscape is altered at this site, however further studies will be needed to investigate this possibility.

While senescence has traditionally been shown to be a response to different forms of cellular stress, recent work has revealed that the senescence program plays critical roles during embryonic development in mice (Muñoz-Espín et al., 2013; Storer et al., 2013; Zhang et al., 2014). To test whether TDG is important during embryonic senescence, I generated a TDG-HET and TDG-KO mouse and assayed for the senescence marker β-galactosidase during embryonic development. Mice which have depleted TDG show perturbed staining at structures that undergo senescence. CDKN2A is not expressed during embryonic development and therefore does not play a role in the establishment of senescence as it does in adult fibroblasts. This finding is consistent with reports highlighting that many of the key players in the senescence of differentiated tissue are likely not drivers of the developmental senescence program (Campisi and D'Adda Di Fagagna, 2007; Collado et al., 2007). Indeed, investigation into the ectodermal ridge, a structure which undergoes senescence at approximately E10.5 revealed that while a subset of known senescence markers were expressed, important markers like p53, p19, or CDKN2A were not expressed (Storer et al., 2013). Gene-enrichment analysis on the set of

genes that are differentially regulated between TDG-WT and TDG-KO revealed a significant enrichment for the categories, “Oxidative Stress Induced Senescence” and “Senescence Associated Secretory Phenotype”. Closer inspection of the genes which underlie these subsets finds that the major statistically significant positive hits are due to a differential expression of histones between the two cohorts. More specifically, histone transcription of various histones appears to be downregulated in TDG knockout in mice: of the 66 genes which are downregulated in our TDG knockout mice, 29 (44%) are histones (Table 3-1). Limited information is available regarding histone dynamics during senescence. Certain levels of certain histone variants like linker histone H1 have been shown to decrease during certain forms of senescence, while other variants appear to decrease (Contrepois et al., 2017; Funayama et al., 2006). Levels of histone marks also change, but the directionality of the changes is believed to dependent on the ‘type’ of senescence program initiated (Parry and Narita, 2016). Determining whether histone dysregulation is important for embryonic senescence will require additional studies in order to fully understand the significance of these observations.

Observing differences in the expression of senescence-associated terms and proteins, even though our RNA-Seq was performed on the entire embryo rather than limited to the cells which undergo differential senescence, suggests that TDG may ‘prime’ cells for senescence *en masse*, which allows for cell-type specific factors to drive senescence in certain structures and not others. Further work, including RNA-Seq targeted at differentially senesced structures in TDG-WT and TDG-KO mice is necessary to determine whether this is indeed the case.

Taken together our findings are the first to show that TDG is general mediator of senescence and, importantly, is able to drive senescence through distinct pathways. Indeed, TDG is one of the few molecules which appear to drive senescence programs in both development and adult tissue. While manipulation of the senescence program is being explored as a therapeutic strategy to overcome various pathologies, major hurdles remain. One of the biggest issues is that the effects of senescence appear to have both beneficial and/or detrimental effects depending on context. For example, the initiation of senescence in tumors leads to SASP which recruits the immune system, resulting in tumor regression and preventing tumor re-growth. Paradoxically, in other cases the initiation of senescence and SASP, has also been shown to be tumorigenic, increasing the aggressiveness and rate at which tumors progressed. Whether this is due to a defect in the senescence program, the immune system, or something else entirely, is not clear. To resolve these sorts of questions and identify new therapeutic targets, identifying important factors and the context under which they function will be required.

3.4 Materials and Methods

Cells culture, treatment and transfections

IMR-90 and HFL cells were obtained from ATCC and grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS). Cell were washed with DMEM and treated with H_2O_2 for 2 h at which point they were washed twice and then replaced with DMEM. Stock H_2O_2 (30%) was diluted with media to obtain desired concentration/amount prior to treatment. siRNA treatments were performed using Lipofectamine 2000

(LifeTechnologies) according to the manufacturer's instructions. Cells were treated for siRNA targeting TDG (Dharmacon, M-040666-01) or scrambled siRNA (Dharmacon, D-001210-03) for 24h at which point cells were washed once and media was replaced with fresh DMEM.

Protein extraction and Immunoblotting

Whole cell protein extracts were obtained by harvesting cells in RIPA lysis buffer (50mM Tris (pH 8) 0.15M NaCl, 1% NP40, 0.5% sodium cholate and 0.1% SDS). Cells were incubated on ice for 15min and centrifuged for 15 min at 4°C (~20,000 RCF). Protein concentrations were normalized, and proteins were separated by SDS-PAGE then transferred to PVDF for 1 hr. The PVDF membrane was placed in blocking buffer consisting of PBS, 0.1% Tween-20 and 5% skim milk powder which was also used for primary and secondary incubation as well as washes. All secondary antibodies used are conjugated to horseradish peroxidase (HRP). To visualize proteins, chemiluminescence film (Anersgan Hyperfilm ECL) was exposed to membranes treated for 5min with Luminata Forte Western HRP Substrate (Cat. No. WBLUF0100).

ChIP and analysis

IMR90 cells were transfected with siRNA targeting TDG or control siRNA for 3 d at which point they were treated with H_2O_2 for the time specified. ChIP was performed using a polyclonal affinity purified TDG antibody (Thermo Fisher Cat. PA5-29140) or with a CBP antibody (Santa Cruz (A-22): sc-369), as previously described, with minor alterations (Thillainadesan et al., 2012). Briefly, cells were cross-linked using 1% formaldehyde in PBS for 10 min under shaking at RT. 0.125 mM glycine in PBS was then added for 5min

to quench the reaction. Cells were then washed twice with ice-cold PBS and harvested in 1 ml of ice cold PBS buffer. The cells were then pelleted at 250g for 10 minutes, washed twice with ice-cold PBS (protease inhibitors added), and then lysed using 200 µl of lysis buffer (1% SDS, 50 mM Tris-HCl [pH 8.0], 10 mM EDTA, and protease inhibitors) for 15 minutes on ice. The cell lysates were then sonicated and cell lysate was centrifuged at 15000 rpm for 15 minutes. An aliquot of the supernatant mixture was saved as input DNA, and the remaining lysate was incubated with 5 ug of antibody in 50 µl of protein A/G dynabeads as per instructions. ChIP was performed overnight at 4°C under rotation. The dynabeads were then washed twice with wash buffer I (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl), once with wash buffer II (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 500 mM NaCl), and then with wash buffer III (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]) and twice with Tris-EDTA buffer (pH 8.0). The chromatin was eluted using 150 µl of freshly made elution buffer I (1% SDS, 0.1 M NaHCO3) twice at 65°C for 10 minutes. NaCl was added to the eluates and to input DNA to a final concentration of 0.3 M and both were incubated at 65°C overnight. Immunoprecipitated DNA was purified using QIAquick PCR Purification Kit (Qiagen) and was analyzed by quantitative PCR with the indicated primers, or run on a gel, in technical triplicates and biological duplicates, unless otherwise noted. Primers used for promoter of CDKN2A:

CDKN2A-ChIP-FORWARD: CTGTCCCTCAAATCCTCTGG

CDKN2A-ChIP-REVERSE: ATTCGCTAAGTGCTCGGAGT

Bisulfite sequencing

DNA was extracted from IMR90 cells using Sigma's Genomic DNA extraction kit. DNA was bisulfite-converted using EpiTect Bisulfite Kit (QIAGEN), according to manufacturer's instructions. Primers used for bisulfite sequencing:

CDKN2A-BS-Forward: GTTGGTAAGGAAGGAGGATTGG

CDKN2A-BS-Reverse: CTCTCCAAAAAAAATCCTTTAAAC

β-galactosidase staining

In vitro and *in vivo* β-galactosidase staining of IMR90 or HFL-1 cells and embryos, was done as previously described (Dimri et al., 1995; Keyes et al., 2005) with alterations. Briefly, after treatments media was removed and cells were washed twice with PBS then fixed with 0.2% glutaraldehyde for 5 minutes after which cells were again washed with PBS, and then treated with the staining mix composed of: 1mg/ml X-gal (Wisent), 150 mM NaCl, 2mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 40mM NaPi (Sodium phosphate buffer). Cells were kept at 37C for 20 hr prior to visualization.

Mouse Protocol

All mouse experiments were done in compliance with the Institutional Animal Care and Use Committee guidelines at London Regional Cancer Center at Western University and the University of British Columbia. To generate *Tdg* knockout mice, *Tdg* $^{fl/fl}$ mice were bred with C57BL/6 Cre-deleter mice which excise loxP flanked regions and generated the *Tdg* heterozygous constitutive knock-out mice. These *Tdg* heterozygous constitutive

knock-out mice were bred to create the range of genotypes used for the embryonic senescence staining assay.

Adult (8 weeks old) UBC-cre/ERT2; *Tdg*$^{fl/fl}$ and litter matched Tdg$^{fl/fl}$ controls were intraperitoneally injected with 3 mg TAM daily for 5 days. To assess cre-ERT2 efficiency, the mice were euthanized 4 weeks after TAM injections, tissues were harvested, lysed using RIPA buffer and western blotting performed using an anti-TDG-antibody.

RNA-Seq and analysis

Sample quality control was performed using the Agilent 2100 Bioanalyzer. Qualifying samples were then prepped following the standard protocol for the NEBnext Ultra ii Stranded mRNA (New England Biolabs). Sequencing was performed on the Illumina NextSeq 500 with Paired End 42bp × 42bp reads. De-multiplexed read sequences were then aligned to the Mus Musculous (mm10) reference sequence using STAR (https://www.ncbi.nlm.nih.gov/pubmed/23104886) aligner. Assembly and differential expression was estimated using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/) through bioinformatics apps available on Illumina Sequence Hub. Prior to down-stream analysis, list of differentially expressed genes was filtered to remove genes on X and Y chromosomes to avoid sex bias. Pathway analysis was performed using Reactome Pathway Database (http://reactome.org) using default parameters. Integrative Genomics Viewer (IGV) was used for viewing high-throughput data-files.

Western Blot quantification was done using ImageJ software and default parameters (https://imagej.nih.gov/ij/).

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Chapter 4. General discussion

4.1 Overview

While TDG has been previously implicated in playing a role in ERα signaling, information regarding the specifics of its role have been limited. To extend the role of TDG in ERα signaling, I performed a ChIP-Seq using an antibody targeting TDG in MCF7 cells -/+ E2, followed by bioinformatic analysis and conventional assays. I found TDG was required for β-estradiol induction of certain ERα target genes. Investigation into the mechanism through which TDG exerts its effects revealed that, in response to β-estradiol, TDG localizes predominantly to distal regulatory regions in ERα target genes. Bioinformatic analysis revealed that these regions are bonafide enhancers and occupied by proteins important to enhancer function, including CBP/p300, ERα and RNA Pol II. Using conventional biochemical methods in conjunction with recently developed techniques, I discovered that TDG is important for both the transcription of long non-coding enhancer RNA from targeted enhancers, as well as the 3-dimensional reorganization that brings together the enhancer with the associated promoter of some target genes. Depletion of TDG resulted in disruption of eRNA production, looping and target-gene transcription. Taken together this work extends the role of TDG in ERα signaling and also uncovers a previously unknown role for TDG: as a regulator of eRNA transcription.

In the context of senescence, I discovered that TDG is required for cells to mount a proper senescence response to oxidative stress in the form of H_2O_2. Using conventional biochemical methods along with ChIP and bisulfite sequencing I found that TDG mediates its effects by upregulating CDKN2A-induction. Importantly, TDG depletion did not result in changes in the methylation status of the CDKN2A promoter, suggesting that its impact is independent of its ability to act in the active demethylation pathway. Indeed, ChIP

performed using a CBP-specific antibody found that TDG is required for the recruitment of CBP to the CDKN2A promoter region, suggesting it is its role as a scaffold, rather than a mediator of active demethylation, that is responsible for activating CDKN2A expression. In addition to senescence in adult tissue, I sought to investigate whether TDG played a role in the senescence program initiated during embryonic development. RNA-Seq on whole mount embryos, either wild-type or TDG-KO, revealed that TDG deletion resulted in defects in the developmental senescence program through a CDKN2A-independent mechanism. Gene enrichment analysis comparing TDG-WT to TDG-KO mice revealed alterations to transcript levels of genes important for "Cellular response to stress", "Oxidative Stress Induced Senescence" and "Senescence-Associated Secretory Phenotype (SASP)" and showed that WNT and Hedgehog pathways may be impacted. Taken together this work provides the first evidence that TDG plays a role in both adult and embryonic senescence programs.

4.2 TDG's effects as an oncogene and tumor suppressor.

The question as to whether TDG is an oncogene or tumor suppressor remains elusive. TDG's ability to repair mismatches and improperly modified cytosines suggests that knockout of TDG should result in increased mutations throughout the genome. Knockout studies performed in mouse model systems have found that deletion of MBD4, the functional homologue of TDG, resulted in increased C:T mutations, as well as increased tumorigenic potential when MBD4 knockout mice are bred with mice predisposed to colon cancer (Wong et al., 2002). Furthermore, TDG functions as a co-activator for the

transcriptional activation of a variety of tumor suppressors, including CDKN2B, p21, p53, CDKN2A, and HIC1 (Hassan et al., 2017; Kim and Um, 2008; Thillainadesan et al., 2012). Collectively, this suggests that TDG may behave as a tumor suppressor and its silencing or mutation should be found in cancer. However, a survey of online databases, including TCGA, finds that TDG mutation or silencing does not appear to be wide-spread. Several interesting observations however may explain this apparent discrepancy: Firstly, knockout studies of TDG found no increase in mutation load compared to wild-type mice, suggesting that functional redundancy compensates for any deficiencies in mutation repair imposed by TDG deletion (Cortázar et al., 2011; Cortellino et al., 2011). Secondly, whatever anti-tumor effects TDG may exert through its control of tumor suppressors or otherwise, must be weighed against any pro-selection advantages it may offer. In the case of breast cancer, I found that depletion or knockout negatively impacts ERα signaling, which is reflected in the blunted response to estradiol-mediated induction of ERα target-genes and decreased proliferation in MCF7 cells. ERα signaling is a key component of early breast cancer development and so it stands to reason that cells with a loss of TDG, which mediates ERα signaling, are likely selected against during tumour development. Similar observations have been made in other systems as well. For example, in Wnt-driven colorectal cancer, the authors found that TDG was required for the upregulation of Wnt targets and drove cancer. Furthermore, no homozygous mutations for TDG are observed in CRC – supporting the idea that the presence of TDG is required for the establishment of the cancer in the first place, which 'protects' it from being silenced, even at the expense of tumors retaining the ability to repair mismatched nucleotides, or activate tumor suppressors (Xu et al., 2014).

Additionally, context appears to be a critical determinant of whether TDG acts as a tumor suppressor or oncogene. For example, a conditional knockout of TDG in the intestinal and colonic epithelium of APC^{Min} mice (i.e. mice predisposed to intestinal adenoma formation) results in significantly higher rates of tumor formation in TDG-KO mice as compared to the controls (Xu et al., 2017). Estrogen plays a protective role in colorectal cancers, with increased rates of tumor formation in females who have undergone an ovariectomy (Xu et al., 2017). Comparing the results of ovariectomies in control mice vs TDG conditional knockouts, researchers found that, in contrast to the findings that TDG is a driver of colorectal cancer by upregulating Wnt signaling, the protective role of estrogen is likely to be largely TDG-dependent, supporting a role for TDG as a tumor suppressor. Collectively these findings reflect similar dynamics that are observed in breast cancer where TDG mediates both pro- and anti-tumor effects, further highlighting its pleiotropic nature.

4.3 TDG functions as a scaffold

In my investigation into TDG's role in ER-signaling in breast cancer as well as its role in senescence, I found no evidence for active demethylation when TDG is engaged. More specifically, in both ERα signaling and senescence, I found that TDG localizes to hypomethylated regions. In the case of senescence, I investigated the methylation status of the CDKN2A promoter, to determine whether depletion of TDG resulted in its hypermethylation and silencing of CDKN2A expression. While TDG depletion did result in reduced CDKN2A levels, this was not accompanied by changes in methylation with the promoter remaining in a hypomethylated state. Instead, I found TDG-dependent

localization of CBP to the CDKN2A promoter upon H_2O_2 treatment. Importantly, TDG depletion prior to H_2O_2 treatment significantly attenuates the recruitment of CBP, consistent with other reports that find that TDG acts as a scaffold, recruiting, among other important transcription factors, CBP (Chen et al., 2003; Tini et al., 2002). While this isn't always the case (TDG's glycosylase activity has also been shown to be critical to retinoic acid signaling for example), it appears that TDG's scaffolding capability is more general and is required in order to stabilize necessary complexes to activate transcription, while its glycosylase activity may only be necessary when removal of DNA methylation is required.

In the context of ER-signaling, I found that TDG localizes to enhancers which were hypomethylated. Therefore, I examined the consequences of TDG knock down at two ER/TDG targeted enhancers (TFF1 and GREB1) using bisulfite sequencing. Interestingly, I found no change in the methylation status of these enhancers, which was not entirely unexpected as previous reports showed that TDG knockout in mouse embryonic stem cells results in the accumulation of active-demethylation metabolites, rather than outright methylation (Raiber et al., 2012; Shen et al., 2013). To interrogate changes to active demethylation metabolites during TDG depletion, I used MAB-Seq. Remarkably, I found that regions bound by TDG are composed almost entirely of unmodified cytosines, and this remained unchanged regardless of E2 treatment and/or TDG depletion. While the lack of methylation at these sites explains why there is no accumulation of active demethylation metabolites in the absence of TDG (methylation must be present in order for active demethylation metabolites to accumulate), it is not clear as to what is preventing DNMT mediated methylation of these specific site yet allowing it to occur at other sites throughout the genome.

4.4 Future Directions

During the course of my investigations, I discovered that TDG plays a critical role in H_2O_2-induced senescence in human fibroblasts and during development. I found that TDG drives H_2O_2-induced senescence likely through the recruitment of coactivator CBP to the CDKN2A promoter. Importantly, while TDG appears to be important for developmental senescence, this occurs independent of CDKN2A, which is not expressed in either TDG-WT or TDG-KO mice. Whole-mount RNA-Seq comparing TDG-KO and TDG-WT mice identified differentially expressed genes between the two cohorts, and gene enrichment highlighted dysregulated pathways which may be responsible for the observed defects. One concern with whole mount RNA-Seq is that it may mask subtle but critical differences that are limited to specific structures that undergo senescence. It will therefore be important to conduct an RNA-Seq on a structure (ex. the apical ecto-dermal ridge) that undergoes differential senescence in TDG-KO vs TDG-WT mice to formally identify genes which are dysregulated upon TDG knockout and which may therefore contribute to defects in embryonic senescence.

While senescent cells have classically been characterized according to phenotypic traits and molecular markers, it has become increasingly clear that cellular senescence can take on many distinct forms depending on cell type as well as the stressors involved in inducing the senescence program. As therapeutic approaches are being developed and tested, classifying different forms of senescence will be important when seeking to effectively target treatments. The role of TDG in H_2O_2-induced senescence and in embryonic

senescence, appears to involve distinct pathways. This suggests that TDG may underlie other forms of senescence as well. A comprehensive survey of the literature to identify different forms of senescence in conjunction with siRNA mediated TDG depletion studies can answer whether or not this is the case. A resource such as this would not only help characterize senescence (helping to establish whether certain proteins or pathways underlie senescence in a general sense or identify critical differences between different senescence programs) but would be useful for identifying new therapeutic targets.

During my research into estrogen signaling I uncovered a novel role of TDG in gene transcription, finding that in response to β-estradiol, TDG mediates the transcription of eRNA from enhancers of ERα target genes. Depleting cells of TDG not only inhibited the transcription of the eRNA's, but also inhibited the transcription of the primary transcripts themselves. Furthermore, eRNA *per se* has been shown to mediate 3-dimensional re-organization at ERα target genes (Li et al., 2013). I confirmed that TDG depletion not only leads to a loss of eRNA production but ablates the 3-dimensional re-organization important for bringing into proximity the promoter, enhancer and all of the transcriptional machinery that resides on both regulatory regions, at the ERα target GREB1. While I found that TDG regulates the production of eRNA from specific enhancers and mediates looping at one of these targets, it is currently unclear how prevalent this is in ERα signaling. To obtain a better understanding of the importance of TDG in ERα signaling, one can use RNA-Seq which gives an accounting of global transcript levels, in conjunction with siRNA-mediated knockdown studies. Alternatively, global run-on sequencing (GRO-Seq) can be used which accurately depicts nascent transcription, to determine the global role of TDG in both eRNA transcription as well as mRNA transcription. In conjunction with insight into how TDG

alters transcription of eRNA and mRNA, tools and technologies such as chromatin conformation capture sequencing (Hi-C) would allow us to determine 3-dimensional chromosome organization and how TDG impacts changes. Integrating this new data with our ChIP-Seq, would provide a detailed overview the role of TDG in ERα signaling.

Interestingly, in many studies approximately the same number of genes are repressed upon E2 treatment as are activated (Osmanbeyoglu et al., 2013). The mechanism through which repression occurs is not clear. However recent work has revealed 3-dimensional reorganization may govern E2-mediated repression (Osmanbeyoglu et al., 2013). Importantly, E2 treatment in MCF7 cells disrupts the 3-dimensional organization at a subset of genes, leading to their repression, while concurrently establishing 3-dimensional contacts at a new set of genes that then become actively transcribed. The role of enhancers and enhancer RNA has not been investigated in the E2-mediated repression of genes, however it seems plausible that the mechanisms that govern formation of 3-dimensional organization in response to E2 treatment at E2 inducible genes also govern the transcription of E2-repressed genes, prior to E2 treatment. Whether TDG plays a role in E2-mediated repression is unknown. To explore the possibility that TDG mediates E2-repression it may be worthwhile to compare our ChIP-Seq data with available datasets in order to establish TDG whether TDG is present at sites which are actively transcribed and then become repressed in response to E2.

Another question that is raised by my work is whether TDG's role at enhancers extends to other signaling pathways such as, TGF-β signaling, RAR-signaling. Indeed, recent work by our lab in retinoic acid signaling observed that in response to retinoic acid, TDG activated transcription of HIC1, an RAR-target gene, through 3-dimensional re-

organization bringing together a distal element with the promoter (Hassan et al., 2017). Whether eRNA production from the distal region mediates this interaction and what role, if any, TDG plays in these dynamics has not been investigated. In HaCat cells for example, TDG activates CDKN2B in response to TGF-β through the active demethylation of the CDKN2B promoter (Thillainadesan et al., 2012). Importantly, loss of TDG results in repression of the promoter and an inability to activate CDKN2B in response to TGF-β. Extending these results globally, using ChIP-Seq, found that that the demethylation of the CDKN2B promoter is unique, as the majority of the overlap between TDG binding and demethylation in response to TGF-β actually occurs intergenically, outside of annotated promoters. In light of recent findings that TDG mediates transcription or active demethylation at enhancers, it would be interesting to explore dynamics at these distal sites in HaCat cells that undergo TDG binding and demethylation. Unlike enhancers in MCF7 cells which are hypomethylated prior to TDG localization, distal sites in HaCat cells are methylated and, concurrent with TDG binding, undergo demethylation. Whether these are enhancer sites that produce eRNA and undergo 3-dimensional reorganization remains to be explored. If these sites are bonafide enhancers, they will provide us with an ideal system to explore the role of TDG-mediated active demethylation at enhancers: determining if its required for coregulator recruitment, eRNA production and 3-dimensional reorganization.

Finally, a key finding of my work was the observation that in the MCF7 breast cancer cell line, TDG has both pro- and anti-tumor functionality: mediating ERα signaling while limiting migration and invasiveness. In the pursuit of identifying therapeutic targets, this mutually-opposed pleiotropy poses an interesting challenge, namely, identifying in what contexts inhibition of TDG may prove to be potentially therapeutic. While empirical tests

employing a wide variety of cell-lines and/or human tissues may be helpful, a global accounting of transcription profiles using RNA-Seq across a wide range of samples can expose which targets of TDG are down-regulated during TDG silencing, allowing for the development of 'markers' than can indicate under which circumstances targeting TDG can be effective.

Collectively, this body of work has expanded our knowledge regarding the mechanism through which TDG functions and extended its role to senescence in adult tissue and embryonic development. Future work extending from this research offers an exciting possibility to deepen our understanding of early transcriptional events that are often dysregulated in various human pathologies and as such may open new targets for therapeutic intervention.

4.5 References

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Zhang, K., Chen, C., Liu, Y., Chen, H., and Liu, J.P. (2014). Cellular senescence occurred widespread to multiple selective sites in the fetal tissues and organs of mice. Clin. Exp. Pharmacol. Physiol. *41*, 965–975.

Zheng, R., and Blobel, G.A. (2010). GATA Transcription Factors and Cancer. Genes Cancer *1*, 1178–1188.

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Curriculum Vitae

BART KOLENDOWSKI

EDUCATION

2012 – Current	**PhD Candidate**, Department of Biochemistry, The University of Western Ontario and Department of Oncology at The London Regional Cancer Program. Supervisor: Joe Torchia, PhD.
2008-2012	**BMSc** (Honors Specialization – *with distinction*)
	Biochemistry & Cell Biology, The University of Western Ontario.

PEER-REVIEWED PUBLICATIONS

1. **Kolendowski, B.**, H. Hassan, M. Isovic, M. Krstic, G. Thillainadesan, A. Chambers, A.B. Tuck and J. Torchia Genome-wide Analysis Reveals a Role for TDG in Estrogen Receptor-mediated Enhancer RNA Transcription and 3-Dimensional Reorganization. **Epigenetics Chromatin**. Jan 29, 2018; 11(1):5. doi: 10.1186/s13072-018-0176-2.

2. *Gameiro S., **B. Kolendowski** (***co-first author**), A. Zhang, J. Barrett, A. Nichols, J. Torchia and Joe Mymryk. Human papillomavirus infection dysregulates the cellular apparatus controlling the methylation status of H3K27 in different human cancers to consistently alter gene expression regardless of tissue of origin. **Oncotarget.** 2017; 8:72564-72576.

3. Hassan, H., **B. Kolendowski,** M. Isovic, M. Underhill, and J. Torchia. TDG is essential for retinoic acid induction of the HIC1 tumor suppressor gene. **Cell Reports**. May 23, 2017; 19(8):1685-1697.

<u>Manuscript in revision or preparation:</u>

4. **Kolendowski B.**, Coughlan N (co-first author), Ziegler K.A., Isovic M., Thillainadesan G., Underhill D.A. and J. Torchia. Global Analysis of CARM1 binding reveals diverse roles in gene regulation. **Oncotarget.** (In revision)

5. **Kolendowski, B.,** H. Hassan, M. Isovic, and Joe Torchia. Role of Thymine DNA Glycosylase in Senescence. In preparation for submission to Mol Cell Biol.

6. **Kolendowski, B.,** M. Isovic, and Joe Torchia. TGF-beta mediated changes to Thymine DNA Glycosylase recruitment and DNA methylation patterns. In preparation for submission to BMC Res Notes.

7. Krstic M, **Kolendowski B**, Cecchini M, Andrews J, Williams K, Leong HS, Torchia J, Chambers AF, Tuck AB. "TBX3-induced EMT of breast cancer cells involves activation of SLUG and MMP14 which is required for increased invasiveness". Manuscript in Preparation.

NATIONAL AND INTERNATIONAL PRESENTATIONS

1. **Kolendowski B**, Hassan H, Isovic M, Torchia J. "The Role of Thymine DNA Glycosylase in estrogen dependent signalling." Keystone Symposia on Nuclear Receptors: Full Throttle. Snowbird Resort, Utah, USA. 2016. (Oral presentation - **Abstract selected for oral presentation**)

2. **Kolendowski B**, Hassan H, Isovic M, Torchia J. "The Role of Thymine DNA Glycosylase in estrogen signalling and breast cancer." CIHR National Student Research Poster Competition. University of Winnipeg, Manitoba. 2016. (Poster)

3. **Kolendowski B**, Hassan H, Isovic M, Torchia J. "The Role of Thymine DNA Glycosylase in estrogen dependent signalling." Keystone Symposia, Nuclear Receptors: Full Throttle (Poster) 2016.

NOTABLE AWARDS

Selected for the CIHR National Student Research Competition June 2016 for the top 5% of graduate students as chosen by their institution, "The Role of Thymine DNA Glycosylase in estrogen signalling and breast cancer." University of Winnipeg, Manitoba. 2016.

Artifical Intelligence Open Grant Competition FINALIST (Top 10%, 450 total applicants)

"Using machine learning to classify and predict drug resistance based on next generation sequencing." (https://aigrant.org/). May 2017.

Best poster presentation award

Kolendowski B, Hassan H, Isovic M, Torchia J. "The Role of Thymine DNA Glycosylase, DNA Demethylation and Enhancer RNA production in estrogen mediated signaling." Oncology Research & Education Day 2015, London, Ontario. 2015. (Poster)

MONETARY AWARDS & SCHOLARSHIPS

2016-2017	CIHR and Translational Breast Cancer Studentship, Breast Cancer Society of Canada **($18,000)**
2016-2017	Schulich Graduate Scholarship **($2,000)**

2016-2017 Western Graduate Research Scholarship **($3,000)**

2015-2016 CIHR - Strategic Training Program in Cancer Research and Technology Transfer **($18,100)**

2015-2016 CIHR and Translational Breast Cancer Studentship, Breast Cancer Society of Canada

2015-2016 Schulich Graduate Scholarship **($2,000)**

2015-2016 Western Graduate Research Scholarship **($3,000)**

2014-2015 CIHR - Strategic Training Program in Cancer Research and Technology Transfer **($18,100)**

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2014-2015 Schulich Graduate Scholarship **($2,000)**

2013-2014 Western Graduate Research Scholarship **($3,000)**

2013-2014 Strategic Training Program in Cancer Research and Technology Transfer, CIHR **($18,100)**

2013-2014 Western Graduate Research Scholarship **($4,500)**

2012-2013 Western Graduate Research Scholarship **($4,500)**